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Natarajan Amaresan
Prittesh Patel
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Practical Handbook on Agricultural Microbiology

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Practical Handbook on Agricultural Microbiology

Edited by

**Natarajan Amaresan, Prittesh Patel,
and Dhruti Amin**

Uka Tarsadia University, Surat, Gujarat, India

 **Humana Press**

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Preface

Agricultural Microbiology is a part of the microbiology branch dealing with beneficial or harmful microbes associated with either plants or soil. This manual focuses on beneficial microbes dealing with soil fertility, microbial degradation of organic matter, soil nutrient transformations, and biocontrol agents. Nowadays, techniques involved in the study of beneficial microbes in agricultural microbiology toward enhancing global agricultural productivity are in trend. This manual covers a wide range of basic and advanced techniques associated with research on the isolation of agriculturally important microbes, identification, biological nitrogen fixation, microbe-mediated plant nutrient use efficiency, and biological control of plant diseases and pests. Introduction to each protocol explains the role/importance of chemicals involved, uniqueness, and protocol application. A proper understanding of the protocol helps the researchers to manipulate them as per their need.

This book is composed of seven parts with 52 protocol chapters. Parts I and II represent the importance, isolation, and purification methods of agriculturally important microbes and include mineral-solubilizing microbes. Part III deals with phytohormones quantitative protocols directly or indirectly associated with microbes. Parts IV and V provide deep insights into protocols for screening agriculturally important enzymes and compounds related to biocontrol activity. Part VI represents assessment methods of soil microbial activity by soil respiration. The final Part VII deals with protocols for selecting microbial strains for inoculant production and quality control ultimately representing commercial biofertilizers production criteria. This book will help postgraduate students, research scholars, postdoctoral fellows, and teachers belonging to different disciplines of Plant Microbiology and Pathology. Moreover, this manual may also serve as a textbook for undergraduate courses like Techniques on Plant-Microbe Interaction/Biological Control of Plant Diseases/Nutrient Use Efficiency.

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Part I

Isolation and Identification of Agriculturally Important Microbes



Chapter 1

Methods for Isolation and Identification of Rhizobia

Vrutuja Naik and Praveen Rahi

Abstract

Rhizobia includes all bacteria that induce nodule formation on the roots of legume plants (*Fabaceae*) and fix atmospheric nitrogen for the host plant in exchange for carbon sources supplied by the plant. In addition to rhizobia, several other bacteria play an important role in the global ecology of nitrogen fixation, and the legumes and rhizobia symbiosis is by far the most important from an agricultural perspective. Being such an important symbiosis, rhizobia research has been initiated in the early twentieth century, which includes isolation and identification of rhizobia. With the advancement of modern molecular biology, rhizobia research also advanced and techniques like MALDI-TOF MS and next-generation sequencing are being used to unravel rhizobial diversity. In this chapter, we discuss various strategies used for the isolation of rhizobia and different identification techniques.

Key word Rhizobia, MALDI-TOF MS, Housekeeping genes, Nitrogen fixation, Symbiosis

1 Introduction

The legume-nodulating bacteria, collectively called rhizobia, form a group of nitrogen-fixing bacteria inside root and stem nodules belonging to the α -subclass and β -subclass of the *Proteobacteria* [1, 2]. The earlier taxonomy reveals that there is wide diversity at the genus, species, and intraspecies levels. Some phylogenetic studies have shown that based on a varied range of legume species, there are many genera of *Alphaproteobacteria* (*Rhizobium*, *Bradyrhizobium*, *Mesorhizobium*, *Ensifer*, *Methylobacterium*, *Devosia*, *Azorhizobium*, *Allorhizobium*) and two genera of *Betaproteobacteria* (*Burkholderia* and *Cupriavidus*) [3–6]. They form a symbiosis with the legume roots and are responsible for the formation of a new plant organ, called root nodules [7]. Rhizobia fix atmospheric nitrogen into ammonia that can be directly consumed by the plant (Fig. 1). They provide atmospheric nitrogen to the plants and in turn plants provide habitats and nutrients. These plant–microbe interactions also play an essential role in disease suppression in plants, resistance to abiotic stress, and protect plants from

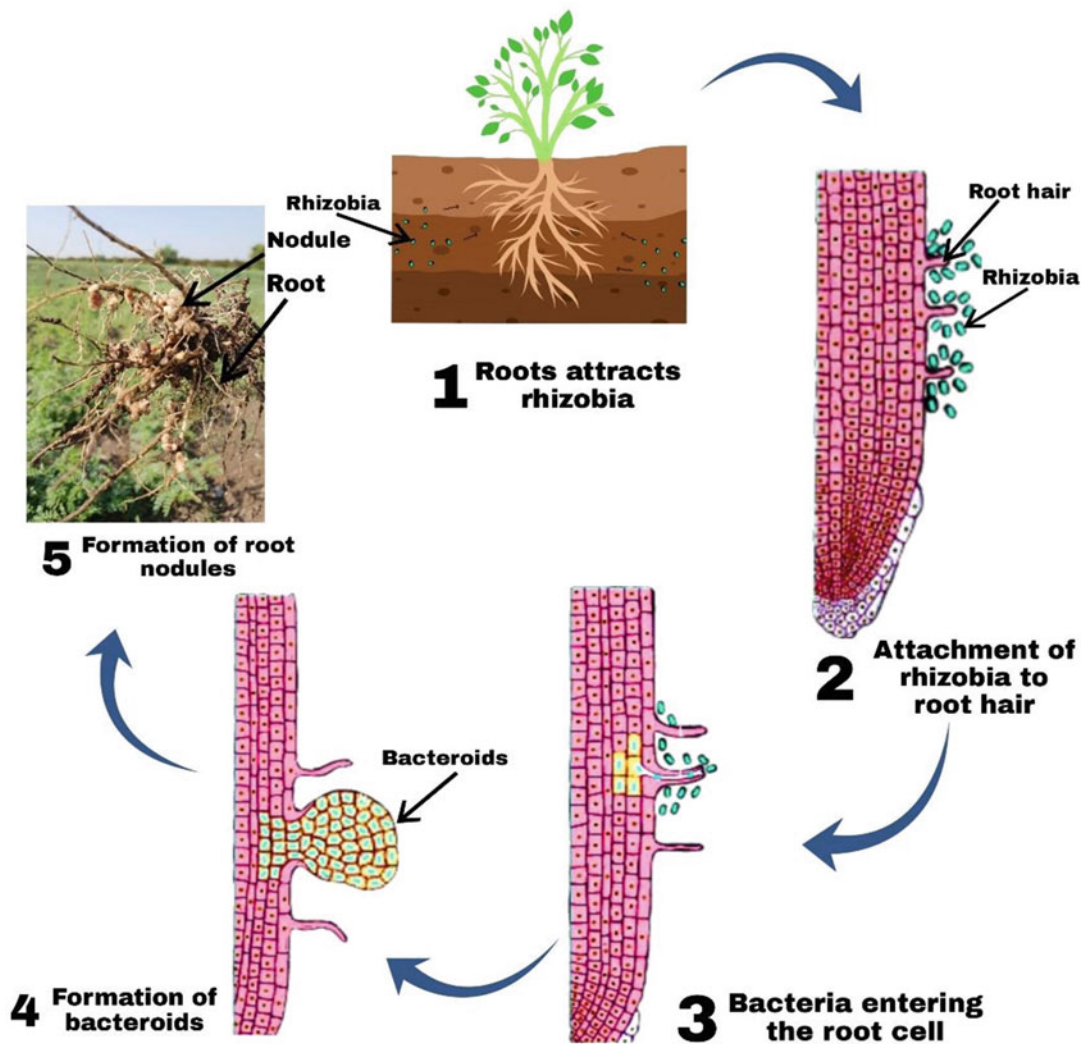


Fig. 1 Formation of root nodule: The leguminous plants release flavonoid which attracts rhizobia. The rhizobia make the root hair curl and gradually form an infection thread which confers rhizobia to enter into the root cells. Rhizobia multiply inside the root cells and form bacteroids, which stimulate cell division of root cells to form root nodules

pathogens [8]. Diversity of rhizobia plays a significant role in identification of bacterial isolates that maximize legume crop productivity. The nodules (particularly those collected from the field) are not always occupied by a single rhizobial isolate nor even by a single microorganism. Nodules of pea and lupin contain both the nitrogen-fixing symbiont and associative organisms such as *Micromonospora* [9].

Earlier, traditional methods were used for the identification of rhizobia. Identification was done on the basis of phenotypic characteristics like shape, color, size, odor, motility, and gram nature.

The API strips are used for identification of microorganisms [10]. Many serological techniques are used for the identification of microbes. Enzyme-linked immunosorbent assay (ELISA) is an analytical technique used for the identification of rhizobia strains [11, 12]. Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) is a technique which is used to analyze proteins. Roberts and his colleagues used two-dimensional PAGE to identify and classify the rhizobia strains based on the patterns obtained on the gel after electrophoresis [13].

MALDI-TOF MS (matrix-assisted laser desorption/ionization time of flight mass spectrometry) is one of the techniques used as a high throughput and reliable method for the identification of bacteria [14]. To study the revolutionary relationships among bacteria, 16S rRNA gene sequencing is the most useful technique [15]. One of the most important advantages of the 16S rRNA region is that, due to its highly conserved region, it supports well-established subdivision of rhizobia into species and genera. The phenotypic characterization can be done by using modified media using varied composition to grow rhizobia under different conditions [16]. The genotypic characteristics can be studied using BOX-PCR [16], ERIC-PCR [17], amplified ribosomal DNA restriction analysis (ARDRA) [18], and restriction fragment length polymorphism profile analysis [19]. The phylogeny and genome sequence study based on housekeeping gene (*atpD* and *recA*) sequences can also be used to determine the relationship among different strains of rhizobia [17, 20, 21].

2 Materials

2.1 Collection of Sample

1. Shovel.
2. Sterile polythene bag.
3. Phosphate buffer with 20% glycerol.
4. Sterile 50 ml tube.

2.2 Isolation

1. Healthy root nodules.
2. Sterile blade.
3. Sterile forceps.
4. Sterile distilled water.
5. Alcohol jar.
6. 4% sodium hypochlorite.
7. 70% ethanol.

8. Sterile YEMA plates (yeast extract 1 g, mannitol 10 g, dipotassium phosphate 0.5 g, magnesium sulphate 0.2 g, sodium chloride 0.1 g, Congo red 0.025 g, and agar 20 g. Final pH (at 25 °C) 6.8 ± 0.2).
9. Sterile loop.
10. 0.85% saline solution.

2.3 Characterization Based on Physiological Features

1. Sterile YEMA plates.
2. Sterile YEMA media without mannitol.
3. NaCl (sodium chloride).
4. HCL (hydrochloric acid).
5. NaOH (sodium hydroxide).
6. Carbohydrates (glucose, sorbitol, sucrose, fructose, malic acid, arabinose, inositol, maleic acid, glycerol, xylose, galactose, mannose, maltose, inulin, starch, dextrose, dextrin, and nicotinic acid).
7. Millipore membrane filter.

2.4 MALDI-TOF MS– Based Identification

1. 150 µl distilled water.
2. 1.5 ml Eppendorf tube.
3. Absolute alcohol.
4. Vortex.
5. Centrifuge.
6. 70% formic acid.
7. Acetonitrile solution.
8. MALDI plate.
9. 11–16 mg/ml HCCA (α -cyano-4-hydroxy-cinnamic acid) solution.
10. MALDI-TOF MS.

2.5 Identification Based on Genomic Features

1. Refer to CTAB chloroform–phenol standard protocol for extraction of DNA.
2. PCR reagents as per standard protocol.
3. Thermocycler.
4. GelDoc.
5. Gel electrophoresis apparatus and reagents as per standard protocol.
6. Sanger sequencing reagent as per standard protocol.

3 Methods

3.1 *Sample Collection*

3.1.1 *Collection of Roots with Nodules*

The best stage to collect roots with nodules is the late vegetative phase (early flowering). Select a healthy plant and uproot it with the help of a shovel if collecting samples from the field. If the samples are collected from a plant grown in a pot, the plant can be uprooted by gently pulling it out from the pot. Shake the plant roots to remove the loosely adhered soil or potting mixture. Separate the root nodules, which are pinkish in color (indicating the presence of leghemoglobin) carefully with the help of sterile forceps. Transfer the nodules into a tube filled with phosphate buffer with 20% glycerol [18, 21].

3.1.2 *Collection of Soil Samples to Trap Rhizobia*

Collect the soil sample (100 g) with help of a shovel (sterilized by dipping in 70% alcohol) into a sample collection bag. To give a better representation of the field, collect soil samples from three different locations, at least 1 m apart from each, and pool them to make a composite sample. Air-dry the soil samples in the laboratory, and store the samples at 4 °C till the samples were used for trapping experiments.

3.2 *Isolation*

3.2.1 *Trapping Rhizobia*

To trap the rhizobia specific to the legume, mix the collected soil sample (mentioned in Subheading 3.1.2) with sterilized potting mixture, generally used potting mixture is vermiculite:perlite (4:1 w/w). Surface sterilize the seeds of the legume of interest by following the stepwise sterilization procedure. First two washes with sterilized water, followed by 3 min dip in 25% sodium hypochlorite solution, 2 min in 70% ethanol, and finally rinsing with sterile distilled water three times. Incubate the sterilized seeds in Petri plates on presoaked sterilized filter paper at 28 °C. Select the germinated seeds for sowing in the pots filled with soil sample and potting mixture, pots devoid of soil samples or with autoclaved soil samples should be used as an uninoculated control. All the treatments in the experiments should be maintained in triplicates. Water the pots with sterilized water and nitrogen-free Hoagland nutrient solution alternatively. Uproot the plants after 30 days of inoculation and check the nodule formation. Separate the nodules as described in Subheading 3.1.1, and use these root nodules for the isolation of rhizobia.

3.2.2 *Direct Isolation*

Direct isolation of rhizobia can be done on yeast extract mannitol agar (YEMA) with Congo red plates. Prepare serial dilution of the soil samples collected as mentioned in Subheading 3.1.2. Plate 100 µl of the serial dilutions up to 10^{-4} dilution onto sterile YEMA plates and incubate at 28 °C. Observe the plates at 24 h interval up to 1 week. Check the colony morphology and select the isolated colonies with typical rhizobia-like colony morphology,

milky white, translucent, glistening, circular in shape, shiny, and raised. To confirm the culture purity, the bacterial colonies were streaked on fresh YEMA plates multiple times [20].

3.2.3 Isolation from Root Nodule

To isolate rhizobia from the root nodules (mentioned in Subheading 3.1.1), wash the root nodules under gentle flow of tap water (*see Note 1*). Transfer the samples to a Petri dish containing a sterilized filter paper to soak-dry the nodules. Now the root nodules are ready for surface sterilization procedure. To surface sterilize the nodules, give two washes with sterilized water, followed by 3 min dip in 25% sodium hypochlorite solution, 2 min in 70% ethanol, and finally rinsing with sterile distilled water three times. Check the surface sterilization of the nodule by rolling the nodule on a sterile YEMA plate before crushing (*see Note 2*). Incubate the plate at 28 °C for observation up to 2–3 days. To isolate the rhizobia, crush the sterilized root nodule on the YEMA plate with the help of sterilized forceps and streak the white root nodule juice with the help of a sterilized inoculation loop. Incubate the inoculated YEMA plates at 28 °C. Observe plates for the growth of typical rhizobia-like bacterial colonies and confirm the purity of bacterial colonies by multiple streaking.

3.3 Characterization Based on Physiological Features

Prepare YEMA plates with different concentrations (1–10%) of NaCl. Streak the isolated pure cultures on these plates and incubate at 28 °C and check at 24 h interval [19].

3.3.1 Salinity Tolerance

3.3.2 pH Tolerance

Prepare YEMA plates with varying pH concentration. Adjust the pH with HCL or NaOH. Streak the isolated pure cultures on these plates and incubate at 28 °C and check at 24 h interval [22, 23].

3.3.3 Temperature Tolerance

Streak the pure culture isolates on YEMA plates and incubate at different temperatures of 4 °C, 10 °C, 15 °C, 35 °C, 40 °C, 45 °C, 47 °C, and 52 °C and check the plates at 24 h interval [22, 24].

3.3.4 Carbon Utilization

Filter-sterilize the carbohydrates (glucose, sorbitol, sucrose, fructose, malic acid, arabinose, inositol, maleic acid, glycerol, xylose, galactose, mannose, maltose, inulin, starch, dextrose, dextrin, and nicotinic acid) by filtration through Millipore membranes. Add each filtered carbohydrate separately to the liquefied YEMA media without mannitol to make a final volume of 10% to the carbohydrates. Then pour the media in plates and let it solidify. Streak the isolated pure cultures on these plates and incubate at 28 °C and check at 24 h interval [16, 23]. Automated platforms like API 20E and API ZYM systems (07584D and 25200, bioMérieux, France) and Biolog system (OmniLog, Biolog, USA) offer fast and reliable

testing for biochemical characteristics, enzyme activities, and oxidation or reduction of carbon sources.

3.4 MALDI-TOF MS–Based Identification

Identification of rhizobia using MALDI-TOF MS has offered a fast and reliable method for identification and proven better than 16S rRNA gene sequence-based identification [25]. MALDI-TOF MS–based identification requires freshly grown bacterial culture. Take a loopful culture in 150 µl distilled water in 1.5 ml Eppendorf tube. Add 450 µl of absolute (100%) alcohol to the tube. Vortex the mixture and centrifuge at 12,000 rpm for 5 min. Decant the solution without disturbing the pellet and keep for air-drying for 5 min at room temperature. Add 20 µl of 70% formic acid to the tube and vortex gently until the pellet dissolves in the formic acid and a thick white liquid is formed. Incubate the tube for 5 min, and add 20 µl acetonitrile solution to the tube and vortex gently. Centrifuge the tube at 12,000 rpm ($10,000 \times g$) for 2 min. The supernatant contains the extracted protein, and pellet contains the unwanted debris. Place 1 µl of extracted protein on a specific spot on the MALDI plate, and allow it to air-dry at room temperature. Overlay 1 µl of HCCA solution (11–16 mg/ml) on the same spot (*see Note 3*), and allow it to air-dry at room temperature. Place the plate in MALDI-TOF MS and calibrate each target plate first using spectra of reference strain. Generate a good quality spectrum for the rhizobial isolate. The spectra having more than 50 peaks of weight between 2 and 20 kDa and with intensity 10^4 should be considered as good spectra and can be used for further identification [26, 27]. Compare the MALDI-TOF MS spectra of the isolates with the Biotyper software database and in-house database to identify the isolated strain. If the score value is between 2.0 and 3.0, then we get species-level identification, and if the score value is 1.7 to 1.99, then we get genus-level identification accurately. If there are high peaks and score value is not reliable, then the spectra of that isolate are not yet present in the database. If extraction of protein is not performed properly, then we do not get any peaks in the spectra. Recent study has suggested that MALDI-TOF MS can discriminate closely related species of the genus *Rhizobium* [25]. A comparison of MALDI-TOF MS spectral profiles of rhizobial strains belonging to closely related species of *Rhizobium* generated several unique and specific peaks (Fig. 2).

3.5 Identification Based on Genomic Features

Extract the DNA from isolates according to the CTAB phenol–chloroform method's standard protocol (<https://jgi.doe.gov/user-programs/pmo-overview/protocols-sample-preparation-information/jgi-bacterial-dna-isolation-ctab-protocol-2012/>). The 16S rRNA gene sequences of majority of rhizobia showed more than 99% similarity to the closely related members, making it difficult to assign species-level identification [25]. Multiple housekeeping genes, like *atpD*, *recA*, and *gyrB*, have been used as

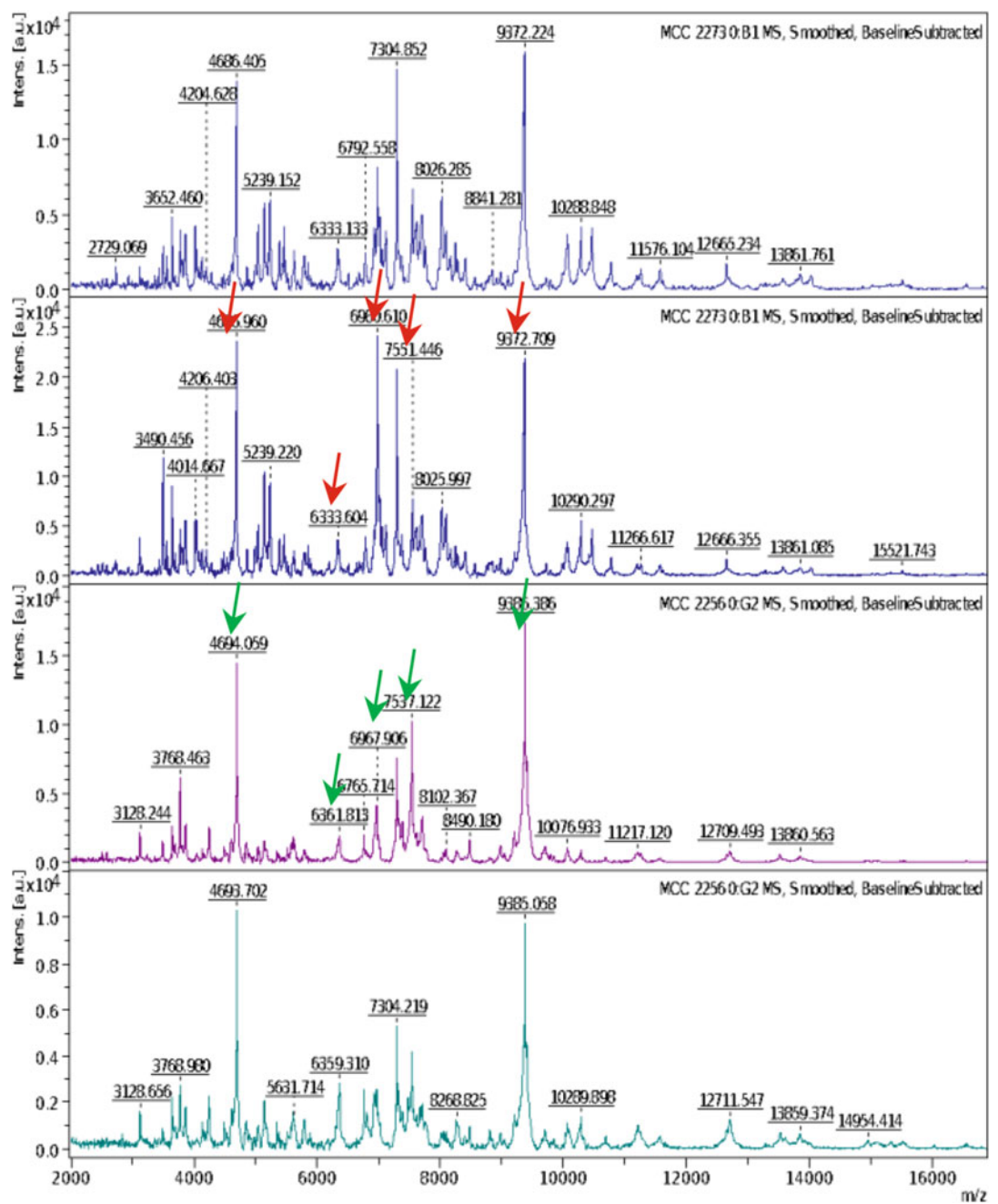


Fig. 2 Comparison of MALDI-TOF MS spectra profiles of two closely related species of *Rhizobium*

an alternative to 16S rRNA gene and DNA–DNA hybridization [28]. A high correlation at the intraspecies level has been observed, on the comparison of housekeeping gene sequence data with DNA–DNA reassociation data. Housekeeping gene sequencing has been considered superior for the assessment of genetic

Table 1
List of primers used for PCR amplification and their sequences

Sr. No.	Target gene	Primer sequences	References
1.	16S rRNA	27 F 5'-AGAGTTTGTATCCTGGCTGAG-3' 1492 R 5'-TACGGYTACCTTGTTACGACT-3'	Lane et al. [30]
2.	23S rRNA	23S11a 5'-GGAAGTGAACATCTAAGTA-3' 23S2205R 5'-CCCAGTCAAACATACCCACC-3'	Van Camp et al. [31]
3.	<i>recA</i>	<i>recA</i> 6F 5'-CGKCTSGTAGAGGAYAAATCGGTGGA-3' <i>recA</i> 504R 5'-TTGCGCAGCGCCTGGCTCAT-3'	Gaunt et al. [32]
4.	<i>atpD</i>	<i>atpD</i> 273F 5'- SCTGGGSCGYATCMTGAACGT-3' <i>atpD</i> 771R 5'- GCCGACACTTCCGAACCNCGCTG-3'	Gaunt et al. [32]
5.	<i>gyrB</i>	<i>gyrB</i> 343F 5'-TTCGACCAGAAYTCTTAYAAAGG-3' <i>gyrB</i> 1043R 5'-AGCTTGTCTTSGTCTGCCG-3'	Martens et al. [28]
6.	<i>rpoB</i>	<i>rpoB</i> 83F 5'-CCTSATCGAGGTTTACAGAAGGC-3' <i>rpoB</i> 1061R 5'-AGCGTGTTGCGGATATAGGCG-3'	Martens et al. [28]
7.	<i>nodC</i>	<i>nodC</i> F 5'-AYGTHGTYGAYGACGGTTC-3' <i>nodC</i> R 5'-CYGGACAGCCANTCKCTATTG-3'	Laguerre et al. [33]
8.	<i>nifH</i>	<i>nifH</i> F 5'-TACGGNAARGSGGNATCGGCAA-3' <i>nifH</i> I 5'-AGCATGTCTCSAGYTCNTCCA-3'	Laguerre et al. [33]

relatedness between the members of rhizobia [28, 29]. Primers used for amplification and sequencing of various housekeeping genes of rhizobia are listed in Table 1. In addition to housekeeping genes, the genes associated with nodulation factors and nitrogen-fixation complexes are also used to understand the symbiotic behavior of rhizobia. The primers used for amplification and sequencing of nodulation and nitrogen-fixation factors are given in Table 1.

To perform PCR amplification for a 25 µl PCR volume, add 2.5 µl of 10x PCR buffer containing 20 mM magnesium, 2.0 µl of 2.5 mM nucleotide mix, 1 unit of Taq polymerase, 1.0 µl of Primer F (Forward) 20 pmol and 1.0 µl Primer R (Reverse) 20 pmol, and 1.0 µl template DNA (10 ng/µl), and make up the total volume to 25 µl with MQ water (*see Note 4*). The PCR amplification for the gene of interest has been performed using specific annealing temperature and up to 35 cycles to get good amplification. Perform agarose gel electrophoresis to ensure the correct gene amplification with reference to appropriate DNA size marker (*see Note 5*). Purify the desired amplified fragments with PEG-NaCl precipitation's standard protocol to remove the remaining unwanted reagents. Perform Sanger's sequencing method of 16S rRNA gene, housekeeping genes such as *recA* and *atpD* and nodulation *nodC*, and nitrogen fixation gene *nifH*. Mix 0.1 µl nuclease-free water,

0.1 µl primer, 0.1 µl template DNA, and 0.2 µl big dye terminator to make a total volume of 0.5 µl.

Align and assemble the sequences generated from Sanger sequencing results using a sequence alignment software like SeqMan. Save the newly aligned sequences as FASTA files for further analysis. Search the sequence as a query at NCBI (National Centre for Biotechnology Information) website, from the “All Resources” tab, select BLAST (Basic Local Alignment Search Tool), and then click on blastn (nucleotide BLAST). To avoid the strains with inaccurate taxonomic assignments, select the check box for “Sequences from type material” and then click the BLAST button.

4 Notes

1. Collect the root nodules that are pink in color and big. The white or shrunk root nodules may not contain the active rhizobia.
2. Use freshly prepared media.
3. Use a freshly prepared HCCA solution in MALDI-TOF MS.
4. Check if any contamination in the PCR reagents before use.
5. Wear a mask while weighing agarose.

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Chapter 2

Isolation of *Frankia* from *Casuarina* Root Nodule

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Abstract

Frankia is a slow-growing, Gram-positive, nitrogen-fixing filamentous actinobacterium that forms a symbiotic association with actinorhizal plants, but it can also be found free-living in soil. In the symbiotic association, *Frankia* is an important contributor to nitrogen fixation. Actinorhizal root nodules will collate and surface-sterilize with 10–20% hydrogen peroxide and the root nodules to crush and dry for dehydrating. One loop of the crushed sample has been directly inoculated on the center of the defined propionate minimal (DPM) medium and broth. The plates and flasks will be inoculated at optimum temperature at 28 °C and allowed 12–15 days for growth budding.

Key words *Frankia*, Root nodules, Nitrogen fixation, Defined propionate minimal medium

1 Introduction

The isolation of *Frankia* has been complicate due to slow growth, high predictability, and unclear growth state of the symbiotic [1, 2]. Recently, *Frankia* has been isolated from nodules of actinorhizal plants by various methods, explicitly (1) sucrose-density fractionation, e.g., *Elacagnus umbellata* and *Alnus crispa*, (2) micro dissection, e.g., *Alnus rubra*, (3) enzymatic digestion, e.g., *Comptonia peregrina*, (4) direct isolation from surface-sterilized pieces of nodules, e.g., *Alnus glutinosa*, (5) Sephadex fractionation, e.g., *Myrica gale* and *Elacagnus umbellata*, and (6) serial dilution, e.g., *Alnus crispa* [3, 4]. *Frankia* is slow-growing, Gram-positive, nitrogen-fixing filamentous actinobacterium that forms a symbiotic association with actinorhizal plants, but it can also be found free-living in soil. In the symbiotic association, *Frankia* is an important contributor to nitrogen fixation [5].

Based on the morphology, chemotaxonomy, and 16S rRNA sequences, the genus *Frankia* is assigned to the phylum actinobacteria and order frankiales. *Frankia* has been subjected to genomic

studies towards its characterization for better understanding and functional role of actinorhizal symbiosis [6]. *Frankia* has a high GC content and grows rather slowly. The symbiosis between actinorhizal plants and *Frankia* induces the formation of a perennial root organ called nodule, wherein bacteria are hosted and nitrogen is fixed. In the field, the actinorhizal nodules can be of various forms and colors [7]. Two types of nodule formation occur in actinorhizal symbiosis: the intracellular and intercellular infections [8]. Symbiotic association of *Frankia* has classified into eight families including *Casuarinaceae*, *Rhamnaceae*, *Betulaceae*, *Datisceae*, *Myricaceae*, *Coriariaceae*, *Elaeagnaceae*, and *Rosaceae*. It is dispersed into 25 genera as well as about 200 angiosperms variety. *Casuarinaceae* is having 4 genera with 96 species: *Ceuthostoma*, *Gymnostoma*, *Allocasuarina*, and *Casuarina*. Among them, *Casuarina* is the majority of broadly planted approximately the worldwide of their habitation of the prefecture [9].

2 Materials

2.1 Root Nodule Sample Collection and Processing

1. Actinorhizal plant *Casuarina* root nodules (fresh sample).
2. Sterile polythene (for sample collection).
3. Methanol.
4. Distilled water (washing purpose).
5. Surface-sterilize agent (15% hydrogen peroxide).
6. Mortar and pestle (nodule grinding purpose).
7. Liquid nitrogen (for making fine residue sample).

2.2 Isolation of *Frankia* from *Casuarina* Root Nodule

1. Defined propionate minimal medium (DPM) (DPM medium composition containing g/L, $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ 1.4 mM, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.2 mM, FeNa EDTA 0.195 mM, KH_2PO_4 5.6 mM, K_2HPO_4 3.2 mM, NH_4Cl 0.5 Mm, pyruvate 1 g, propionate 1 g, salinity 2%, microelements 1 mL (H_3BO_4 , $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$), and vitamins 0.1 g (pyridoxine and biotin) at a final pH of 8.5).
2. Sterile Petri plates.
3. 250 mL Erlenmeyer flask.
4. Antibacterial (streptomycin, ampicillin).
5. Antifungal (cyclohexamide or amphotericin B).
6. Shaker (at 28 °C) and incubator (30 °C).

2.3 Cultural Characterization of *Frankia*

1. 15 days old incubated root nodule sample plates.
2. 7–12 days old incubated root nodule sample Erlenmeyer flask.
3. Light chamber.

2.4 Morphological Characterization of *Frankia*

1. Microscope glass slides.
2. Culture plate and broth.
3. Trypan blue stain (0.5%).
4. Glass coverslip.
5. Inverted phase-contrast microscope.
6. Centrifuge.
7. Critical point drying.
8. Phosphate-buffered saline (PBS, pH 7.4).
9. Karnovsky's fixative solution (2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4).
10. Freeze-dryer.
11. Scanning electron microscope.

2.5 Molecular Characterization of *Frankia*

1. gDNA is extraction by the fine chemicals as per standard protocols of salt-precipitation method.
2. Microcentrifuge tubes.
3. Centrifuge.
4. TKM 1 buffer.
5. TKM 2 buffer.
6. Lysozyme.
7. Proteinase K.
8. Other materials as per standard protocols of salt-precipitation method.
9. PCR fine chemicals as per standard protocols.
10. Primers *msa* F27 (5'-AGAGTTTGATCMTGGCTCAG-3') and R765 (5'-CTGTTTGCTCCCCACGCTTTC-3').
11. Thermocycler.
12. GelDoc/transilluminator.
13. Phylogenetic analysis—Codoncode Aligner v4.1.1. (Sequence contig editing purpose).
14. ClustalW (carries out sequence similarity and multiple sequence alignment).
15. MEGA v6.0 software package with maximum likelihood (ML) algorithm (Tamura-Nei model) (generates phylogenetic trees).
16. NCBI database using Bankit software (gene sequences deposit).

3 Methods

3.1 Root Nodule Sample Preparation for *Frankia* Isolation

1. Collect healthy fresh root nodule in a sterile plastic bag and transfer to the lab.
2. Wash the collected root nodule sample with distilled water to remove dirt and soil from the outer face of the root nodule.
3. Surface-sterilize the fresh root nodule with 15% hydrogen peroxide for 10–15 min to remove the unwanted microorganisms.
4. The surface-sterilized root nodule is dried with the help of tissue paper for 15–20 min to avoid the fungal contamination.
5. After dried, grind the nodules with the help of sterile mortar and pestle.
6. The root nodule is allowed to be ground until the sample is made into fine residue with the help of liquid oxygen.
7. The fine root nodule residue is ready for *Frankia* isolation (Fig. 1) [10].

3.2 Isolation of *Frankia* from *Casuarina* Root Nodule

1. Smoothly inoculate the fine root nodule residue on to the DPM agar medium plates supplemented with antibiotics (streptomycin, ampicillin, and fungicide cycloheximide, 100 µg/mL) [10].



Fig. 1 (a) Root nodules surface-sterilize with 15% hydrogen peroxide (b). Root nodules drying with the help of tissue paper (c). Root nodules grinding (d). Fine root nodule residue

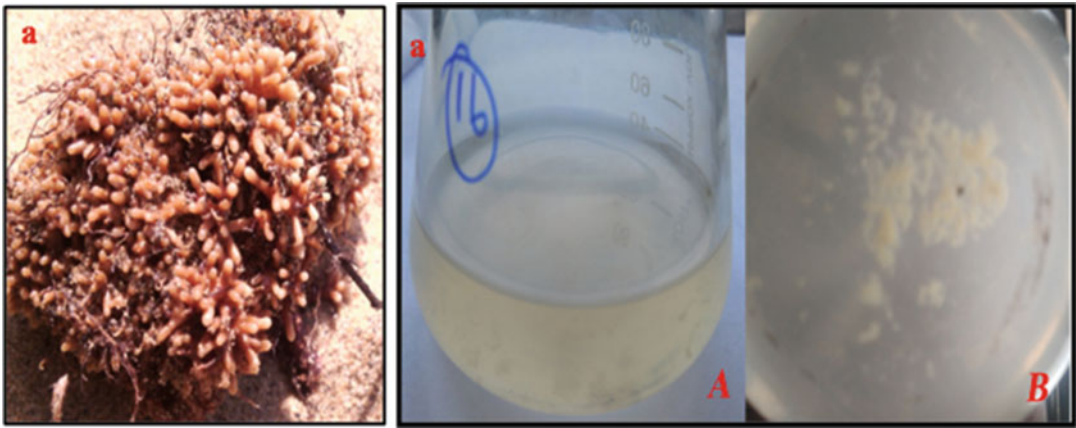


Fig. 2 (a) Root nodules (b) [A]. Broth culture of *Frankia* [B]. Colony zoom photograph of *Frankia*

2. Similarly transfer the fine root nodule residue onto the DPM broth medium along with similar antibiotics.
3. Incubate the plates at 28°C for 12–15 days as they are relatively slow growers (till the distinct colony appears).
4. Purify the colonies from the master plate and flask to store at –80 °C as a glycerol stock (Fig. 2) [11].

3.3 Observations of *Frankia* Isolation

Root nodule and *Frankia* isolation.

3.4 Characterization of *Frankia*

3.4.1 Cultural Characterization of *Frankia*

1. Observe the fast-growing transparent or colorless thread-like fluffy white cloudy colonies that appear on the DPM culture plates after 12 days of incubation.
2. The clear fluffy, chalk-white colonies appeared on the DPM broth culture.
3. Fresh isolated colonies are selected and subcultured on the sterile DPM medium and NA (nutrient agar) for free from contamination.
4. Pure fresh colonies are used for further characterization [8].

3.4.2 Morphological Characterization of *Frankia*

1. Pick out the thread-like fluffy white cloudy colonies and transfer to microscope glass slides with a 0.5% trypan blue stain.
2. Examine slide covers with a glass coverslip in an inverted phase-contrast microscope at 40× and 100× (oil immersion) magnifications.
3. Similarly, in the broth culture, centrifuge the clear fluffy, chalk-white colonies at $7168 \times g$ for 10 min, collect the pellets, wash in sterile distilled water, and homogenize in a mortar and pestle.

4. Examine the cells, hyphae, vesicles, and sporangia in an invert phase-contrast microscope [9].
5. To view the ultrastructure of *Frankia* morphology on scanning electron microscope to take 7 days old *Frankia* cultures and grown in low-phosphate MPN medium supplemented with or without 1 mM CuSO₄ for 3–4 days.
6. After growth, centrifuge *and* harvest cells at $10,000 \times g$ for 10 min and harvest sample process via a critical point drying method [10].
7. Briefly, wash cells three times with phosphate-buffered saline (PBS, pH 7.4), and fix the samples by incubation in modified Karnovsky's fixative solution (2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4) for 4 h.
8. Wash the samples one time with PBS and follow by a distilled water wash.
9. Dehydrate washed fix cells by critical point drying through a series of alcohol dehydration steps (30, 50, 70, 90, and 100%).
10. Cover dehydrated samples with *t*-butyl alcohol for freeze-drying and sputter-coated with gold.
11. View the samples at 1000 to 20,000 $\times$ magnifications on a scanning electron microscope (Fig. 3).

3.5 Molecular Characterization of *Frankia*

1. Extraction of genomic DNA to take the preeminent growth of isolates, and gDNA is extracted by the salt-precipitation method with minor alterations [10].
2. Take 2 mL broth cultures of 4 isolates in microcentrifuge tubes and collect the cells through centrifugation at $11200 \times g$ for 10 min.
3. Collect and wash cells two times with 0.5 mL of TKM 1 buffer monitored by the addition of 0.4 mL TKM 2 buffer and 150 μ L lysozyme.
4. Incubate the content at 37 °C for 45 min with broken fraternization and supplied with 30 μ L of proteinase K.
5. Add proteinase K followed by 30 min incubation at 55 °C in a water bath, and the resulting mixtures supplied with 50 μ L of 10% SDS and 250 μ L of 6 M sodium chloride.
6. Further, centrifuge the tubes at $16128 \times g$ for 10 min and transfer supernatants to fresh microcentrifuge tubes.
7. Add equivalent volume of ethanol to the supernatant and mix gently by inverting the tubes followed by centrifuge at $16128 \times g$ for 8 min.
8. Wash the plates with 70% ethanol and dry the sample at room temperature.

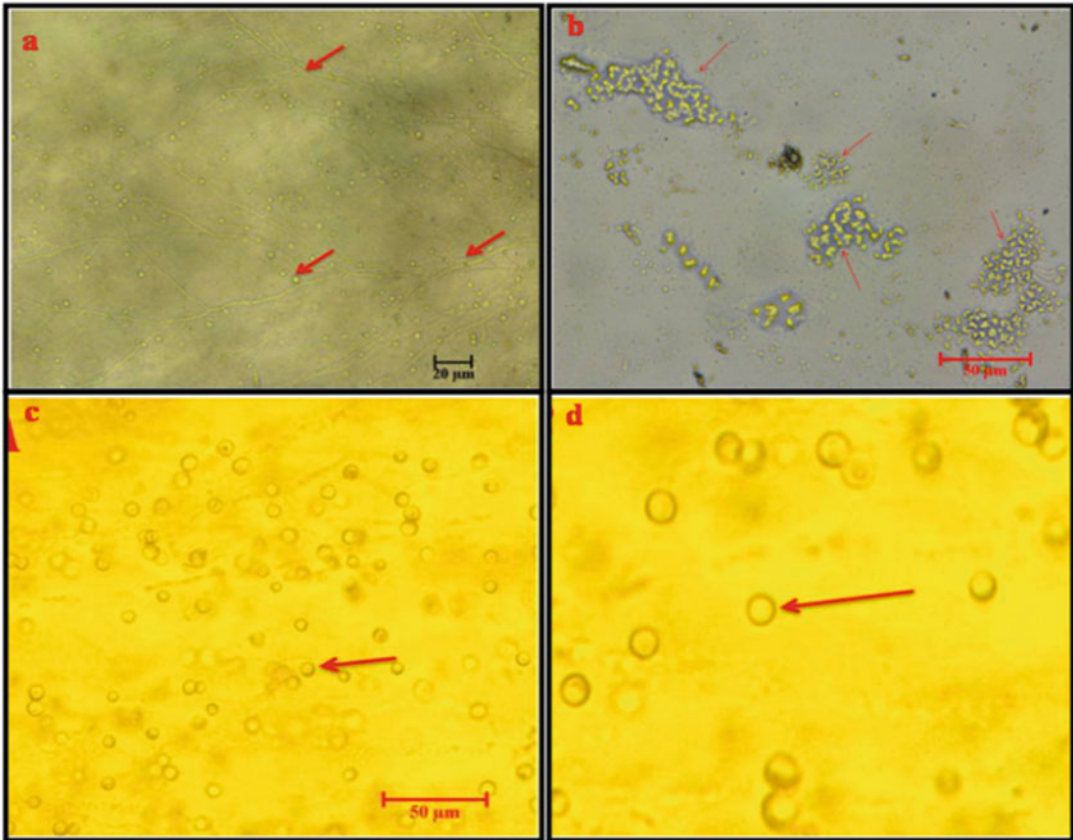


Fig. 3 (a) Light microscopic photograph of *Frankia* with branched septate hyphae with vesicles. (b) Light microscopic photograph of *Frankia* sporangia. (c, d) Fluorescence microscopic photograph shows vesicles structure of *Frankia*.

9. Finally, resuspend the pellet in 50 μL of TE buffer by heating at 65 $^{\circ}\text{C}$ for 15 min and store at -20°C for further analysis.
10. Visualize the quality of DNA extract by gel electrophoresis with 0.8% agarose (w/v) containing 0.5 mg mL^{-1} ethidium bromide at 80 V for 1 h using $1\times$ TAE buffer and observe under gel documentation system (Nyx Technik PhotonyxTM S140, Minnesota, USA).
11. PCR amplification of 16S rRNA gene: the 16S rRNA gene of all the isolates is amplified in an automated thermal cycler (Applied Biosystems, Foster City, CA, USA) using universal 16S primers, F27 (5'-AGAGTTTGATCMTGGCTCAG-3') and R765 (5'-CTGTTTGCTCCCCACGCTTTC-3') [11].
12. PCR is performed in a total volume of 50 μL containing, 25 μL $2\times$ PCR premix, 4 μL template DNA, 3 μL of each primer, and 15 μL of sterile distilled water.
13. The cycling parameters used are as follows: initial denaturation at 94 $^{\circ}\text{C}$ for 8 min, followed by 35 cycles of denaturation at

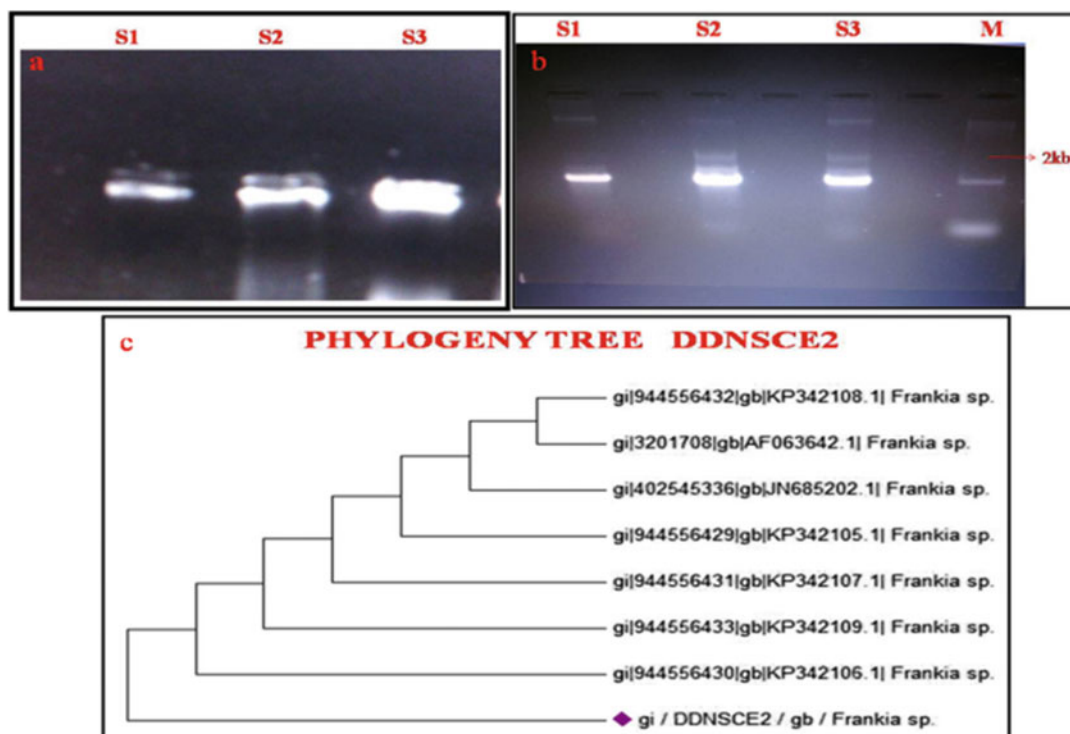


Fig. 4 (a) Separation of gDNA of *Frankia* on 0.8% agarose gel electrophoresis. (b) PCR image of *Frankia* on 1% agarose gel electrophoresis. (c) Phylogenetic tree of *Frankia* using neighbor-joining software

94 °C for 1 min, annealing at 65 °C for 1 min and extension at 72 °C for 2 min, and then a final elongation at 72 °C for 10 min [12].

14. In the end of the PCR, the products are electrophoresed through 1% agarose gel to ensure the quality as well as the purity of amplicons.
15. A mixture of HindIII digested λ DNA and HaeIII digested ϕ X174 DNA is used as a molecular marker (F-303SD, Finnzymes, Espoo, Finland) [8] (Fig. 4).
16. Sequencing and phylogenetic analysis, to check the purity of amplicons by use PCR product pre-sequencing kit and sequence.
17. Electropherogram is acquired, revised, and sequence contig and editing using Codoncode Aligner v 4.1.1.
18. Further, conduct a BLAST search for sequence similarity, and multiple sequence alignment by using ClustalW [11].
19. Concurrently, generate phylogenetic trees using MEGA v6.0 software package with maximum likelihood (ML) algorithm (Tamura-Nei model), where the interior branch lengths test via bootstrap analysis with 1000 replications and partial 16S rRNA

gene sequences deposit in the NCBI database using Bankit software tool available online (<http://www.ncbi.nlm.nih.gov>) (Fig. 4).

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Chapter 3

Isolation and Identification of Nonsymbiotic *Azotobacter* and Symbiotic *Azotobacter Paspali*–*Paspalum notatum*

Bhavana V. Mohite and Satish V. Patil

Abstract

Azotobacter is a renowned nonsymbiotic nitrogen fixer. Since the discovery of *Azotobacter* in 1901, it has magnetized microbiologists' attention for its interesting potential in agriculture for nitrogen fixation as well as synthesis of biologically active substances. It has distinctly enhanced effect on crop production in agriculture by which it deciphers the growing demand of food for ever-increasing population. The exploration of free-living *Azotobacter* sp. along with unique symbiotic *A. paspali* will be an attempt towards augmentation of soil fertility with enhanced crop yield. This chapter will brief the general strategy for isolation and identification of *Azotobacter* with elementary approach.

Key words Cyst, Pigment, Exopolysaccharide, Nitrogen-free medium, Biofertilizer

1 Introduction

Azotobacter belongs to *Azotobacteraceae* family, proteobacteria subclass including nonsymbiotic-free nitrogen fixers and frequently has habitat in soil and water together with sediments [1]. *Azotobacter chroococcum* is the foremost reported species of *Azotobacter* from Holland soil by Beijerinck [2]. Subsequently, various new variety of *Azotobacter* sp. has been reported from soil and rhizosphere.

The *Azotobacter* genus has seven reported species namely *A. chroococcum*, *A. beijerinckii*, *A. vinelandii*, *A. paspali*, *A. armeniacus*, *A. nigricans*, and *A. salinestrus* [3]. The utmost quantity of DNA in *Azotobacter* in comparison with other bacteria may be due to larger cells of *Azotobacter* [1]. The mol% GC content of *Azotobacter* is 52 to 67.5. The amount of DNA and quantity of chromosomes is augmented together with ageing.

NifH gene is expansively sequenced gene used for identification of nitrogen fixing *Azotobacter* [4]. The *Azotobacter* sp. has the metabolic potential of fixation of atmospheric nitrogen into ammonia. The three discrete nitrogenase enzymes, molybdenum

(Mo) nitrogenase, vanadium (V) nitrogenase, and iron-only (Fe) nitrogenases, are the remarkable characteristic to study *Azotobacter* as fascinating nitrogen fixer with its noteworthy agriculture potential.

Azotobacter shows a mixotrophic, autotrophy, or heterotrophic mode of nutrition. The combined nitrogen-free medium with appropriate carbon resource is the ideal prerequisite for the *Azotobacter* growth. The optimum temperature of *Azotobacter* growth is 28–37 °C, but may differ as per the species. The acidic to alkaline pH range beginning from pH 4.8 up to pH 8.5 is applicable for *Azotobacter* growth.

Azotobacter plays a remarkable role among the free-living nitrogen fixing microorganisms as widely distributed in the natural habitat; soil, water, and sediments. The *Azotobacter* has proved as excellent bioinput for crops by nitrogen fixation and affecting the plant growth and yield, producing different plant growth promoting substances as well as stimulating the microflora of the rhizosphere [1].

Most of the *Azotobacter* sp. is from soil, slightly acidic to alkaline soil, but few are from water. The requirement for high phosphorous leads to prevalence mostly in fertile soil. One remarkable sp., *A. paspali*, was isolated from roots system of Bahia grass (*Paspalum notatum* cv Batatais), a tropical grass, due to accessibility of organic substances and appropriate pH by the plant in the rhizosphere. *Azotobacter paspali* grows in the rhizosphere of *P. notatum*, and nitrogen fixed by it may be transferred to Bahia grass and hence improve pasture growth. This restriction to the plant rhizosphere may be due restriction of utilization of wide variety of organic substances. *A. paspali* has the unique antagonistic property against the Gram-positive bacteria which is a beneficial property for life in the rhizosphere. Increases in the nitrogen content of the roots and in the total nitrogen content of the sand plant system were associated with successful *Azotobacter* colonization [5]. The *A. paspali* shows symbiotic highly specific diazotrophic association with *P. notatum* and hence it is interesting to culture and study it.

Azotobacter is straight rods having rounded ends becoming ellipsoidal or coccoid-shaped based on culture age and medium. The dimension is 2 or more $\times$ 4 μ m (diameter $\times$ length). *A. paspali* is generally longer (5 $\times$ 10 μ m length) and filamentous (up to 60 μ m long). The cells are single but can present in pairs or irregular clumps like in *A. paspali*. The morphological type changed according to culture condition. This property along with inability to use several carbon sources, make it a unique species. The increase in carbon: nitrogen (C: N) ratio and culture age [6] causes aggregation of *A. paspali* cells in late logarithmic or stationary phase. *A. vinelandii* and *A. paspali* are the only *Azotobacter* sp. carrying

nif, vnf, and anf genes producing either of the three nitrogenases enzymes based on Mo or V supply in the environment.

Azotobacter is nonmotile or motile with peritrichous flagella. The pigment production and cyst formation are the peculiar characteristics of *Azotobacter* apart from nitrogen fixation potential. The *Azotobacter* sp. undergoes encystment during late stationary stage with production of water soluble or insoluble type of pigments.

1.1 Isolation of the *Azotobacter*

Isolation of *Azotobacter* sp. is practiced using specific designed medium based on its nutrition category as chemoheterotrophs and potential of fixation of dinitrogen. The enrichment technique is principally based on the potential of nitrogen fixation by aerobic/microaerobic way and utilization of organic substrates as energy source. The enrichment can be carried out by addition of specific stimulatory or inhibitory selective substrate into N₂-free medium such as erythritol or D-arabitol, L-rhamnose, ethylene glycol as carbon source for *A. vinelandii*, O-hydroxybenzoate, D-glucuronate or D-galacturonate, and L-tartrate, pH 6 or less for *A. vinelandii*, *A. beijerinckii*, caprylate for *A. armeniacus*, and 35–37 °C temperature for *A. paspali*.

The soil paste plate and silica gel method were conventionally described for isolation of *Azotobacter* sp., in which soil/silica gel is fortified with suitable carbon source and other nutrient elements, seeded with sieved soil and allowed to incubate [3].

The isolation technique comprised of utilization of various nitrogen-free agar medium such as Winogradsky's [7] nitrogen-free media, LG medium [8], Norris medium [9], Ashby's medium [10], and Burk medium [11]. These reported media are fairly similar, merely get differ with a few carbon sources and proportion of micro and macronutrient and minerals (Table 2) (*see Note 1*).

Apart from general N₂-free medium, some medium can be designed to make it selective with addition of particular constituent for particular isolation of *Azotobacter* species. For example, for isolation of *A. paspali*: the sucrose medium can be made selective with addition of 0.5% bromothymol blue and using rhizospheric sample from *Paspalum notatum*, for *A. beijerinckii* using α-hydroxyl benzoate, Tartarate and D-galaturonate with maintaining pH 4.9–5.5, for *A. vinelandii* isolation addition of erythritol, butanol, rhamnose, ethylene glycol, 0.1% phenol, and 10% sodium benzoate, for *A. chroococcum* pH need to be maintained at pH 7.0–7.5, for *A. nigricans* addition of citrate, *n*-valerate and for *A. salinetris* Burk medium is fortified with 1.0–2.0% sodium salt [12].

1.2 Identification of the *Azotobacter*

The organism isolation after the enrichment culturing has increased the possibility of isolation of free-nitrogen fixer. The primary confirmation of *Azotobacter* genus is carried with principal

morphological tests, i.e., cyst formation and pigment production. *Azotobacter* could be differentiated from residual nitrogen fixers based on simple characteristic property of cyst-forming potential.

1.2.1 Cyst Formation

Azotobacter vegetative cells show rods to ovoid shape morphology, and consequently they may also present in larger clumps. In stress condition, the vegetative cells change to round, dormant cell structure referred to as cyst by the process of encystment. The formation of cyst is the leading criteria for taxonomic classification of *Azotobacter* [13]. *Azotomonas* and *Derecio Azomonas* are nitrogen fixers, but do not have the ability to produce cyst. The formation of cyst could be induced by particular carbon sources like ethanol, butanol, β -hydroxybutyrate, and isopropanol as a carbon source [7] (see **Note 2**).

1.2.2 Pigment Production

The diffusible and fluorescent pigment production is characteristic property of *Azotobacter* sp. and can be studied in daylight or under ultraviolet light, respectively. The basal agar media of Thompson and Skerman [14] enriched with sodium gluconate could be used for diffusible pigment while Stainer and Scholte medium is used for the nondiffusible pigment. The colonies of *Azotobacter* appear first white, flat, and mucoid, later on become quite glossy, convex, although the type of medium and carbon sources varies the colony morphology [14]. The further incubation allows pigment production (Fig. 1). The identification of *Azotobacter* sp. could be carried out based on pigmentation type as unique type of pigment produced by specific *Azotobacter* sp. (Table 1).

1.2.3 Identification of *Azotobacter* sp. at Species Level

The cyst formation property on N_2 -free medium like Burk's, Ashby's, and Norris proved that the isolate is belonging to *Azotobacter* genus. The species-level identification is relying on range of phenotypic and biochemical investigation. The use of typical carbon source, type and color of pigment, response to particular antibiotics are basis for species-level identification of *Azotobacter* [12] (see **Note 3**).

1.3 *Azotobacter paspali*

The new species of *Azotobacter* named *Azotobacter paspali* [15] was isolated using the silica gel plates, containing Winogradsky's salts and calcium citrate as a carbon source from the rhizosphere soil of *Paspalum notatum*. This name was later changed to *Azorhizophilus paspali* [16]. The unique characteristic for identification of the *A. paspali* sp. is younger filamentous long rods cells (5–10 μ m length and 1.3–1.7 μ m in width). It produces red violet water-soluble pigment or yellowish-green fluorescent colonies. Döbereiner [15] examined growth of *A. paspali* on N_2 -free modified and Lipman medium [17] having sucrose as solitary carbon source and bromothymol blue indicator.

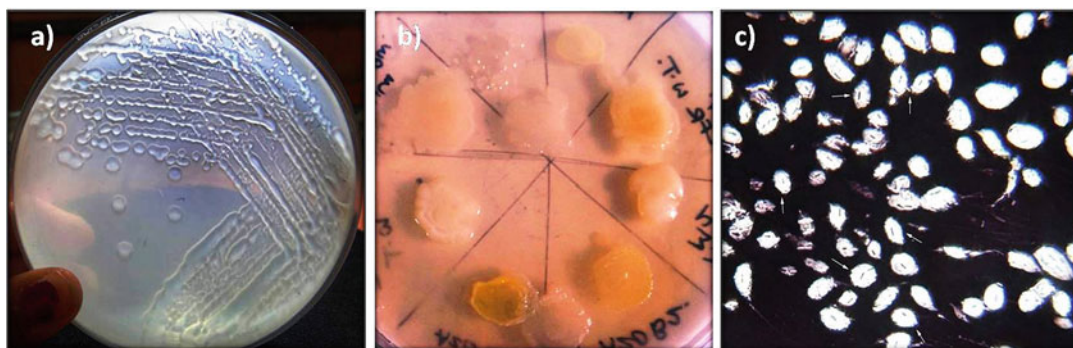


Fig. 1 (a) *Azotobacter* sp. on nitrogen-free medium, (b) Different *Azotobacter* sp. pigment production, and (c) *Azotobacter* with surrounding biopolymer by negative staining

Table 1

***Azotobacter* sp. with its specific type of pigment**

<i>Azotobacter</i> species	Type of pigment
<i>A. vinelandii</i>	Yellow-green, fluorescent, water-soluble pigment
<i>A. beijerinckii</i>	Yellowish or cinnamon pigment
<i>A. paspali</i>	Yellow-green fluorescent or red-violet water-soluble pigment
<i>A. chroococcum</i>	Brown or blackish-brown
<i>A. nigricans</i>	Yellow nondiffusible pigment
<i>A. armeniacus</i>	Diffusible brown-black or red-violet
<i>A. salinetrus</i>	Black-brown

A. paspali produces yellow color on a blue background on a sucrose minerals medium containing bromothymol blue indicator, representing the characteristic property of organic acid production (Table 2). After 48 h of incubation, yellow-centered colonies of *A. paspali* are appeared due to the medium acidification and hence resulting bromothymol blue assimilation. *A. paspali* is motile with peritrichous flagella while some strains have curli flagella. *A. paspali* can produce H_2S from thiosulphate and has potential to grow at $14^\circ C$.

A. paspali has the unique property of definite rhizospheric association with the wild grass; hence, it is considered to represent the symbiotic nitrogen association. *Azotobacter paspali* affects the growth and development of plant by appreciable increase in weight of roots and shoot [18]. *A. paspali* has specificity for a *Paspalum notatum*, a wild grass, along with some additional *Paspalum* sp., i.e., *P. plicatulu* and *P. virgatum* [19].

Table 2
Differential characteristics of *A. paspali*

Characteristic	<i>A. paspali</i>
Motility by peritrichous flagella	+
Cell morphology	+
Cells in pairs, irregular clumps	+
Long filamentous cell	+
Undulate edged unevenly convex colony with rough surface	
Water-soluble pigments	+
Yellow-green fluorescent pigment	
Brown-black to red-violet	+
Nitrogen fixation occurs at pH	—
5.0–5.5	d
6.0	+
6.5–9.5	+
10	
Growth at temperature of	—
9 °C	+
14 °C	+
18 °C	+
32 °C	+
37 °C	
Enzyme production	—
Peroxidase	+
Urease	+
Oxidase	
Production of H ₂ S from	+
Thiosulphate	d
Cysteine	
Utilization of sole carbon source	+
Fructose, glucose, acetate, pyruvate, fumarate, malate, succinate, α -oxoglutarate, lactate,	+
DL gluconate, acetylmethylcarbinol	—
Sucrose	+
Propan-1-ol	
Maltose, trehalose, melibiose, raffinose, mannitol	
Utilization of sole nitrogen source	+
Ammonia	+
Nitrate	—
Glutamate	
Nitrate reduced to nitrite	—
Nitrogen fixation genes	+
Nif	+
Vnf	+
Anf	
Mol % G + C (T_m) ²	63.2–65.6
Genome size	4.3–4.6 Mb

Key: +: Positive, —: Negative, d: variable

2 Materials

1. Soil sample from a fertile soil/macerated roots or leaves or other samples.
2. Sterile saline tubes (10 tubes) for serial dilution of the soil samples.
3. Sterile N₂-free medium liquid enrichment medium (Burk's medium, Sergei Winogradsky's N₂-free medium, LG medium, Ashby's medium, Norris medium, Brown's medium, and Döbereiner sucrose mineral medium).
4. Sterile N₂-free medium agar plates (Burk's medium, Sergei Winogradsky's N₂-free medium, LG medium, Ashby's medium, Norris medium, Brown's medium, and Döbereiner sucrose mineral medium).
5. Stain for cyst: violamine/acridine orange/mixture of neutral red, light green, SF yellowish.
6. 0.5 ml of 10% (v/v) glycerol or paraffin oil or in 7% dimethyl sulfoxide in 0.1% phosphate buffer (pH 7.0) for maintenance of culture.

3 Methods

1. Take 1.0 g of collected soil/macerated roots or leaves or other samples, add in 9.0 ml sterile saline, mix well by shaking, and then further dilute up to tenfold, i.e., 10⁻⁹.
2. Prepare N₂-free medium (Burk's N₂-free medium, Sergei Winogradsky's N₂-free medium, LG medium, Ashby's medium, Norris medium, and Brown's medium) in pure distilled water in clean 250 ml Erlenmeyer flasks and sterilize it (121 °C for 20 min at 15 psi).
3. The aliquots of diluted suspension (use last 3–4 dilutions, 10⁻⁶ to 10⁻⁹) are incubated in liquid N₂-free agar medium (24–48 h at 30 °C). After the appearance of macroscopic growth, the culture is observed microscopically and cell morphology is observed.
4. The positive cultures are streaked from that liquid medium to the surface of specific N₂-free medium agar plates with selective substance.
5. After incubation of 24–48 h, colonies will appear; further allow it to incubate for 3–5 days to observe the diffusible and fluorescent pigment production and about 2 weeks for cyst formation.

6. Cultures grown for 24–48 h on liquid and solid medium are studied for general morphology by microscopic observations like Gram staining, motility, and cultural characteristics.
7. After incubation of 3–5 days, the plates are observed for pigment production (diffusible pigment in daylight and fluorescent pigment under ultraviolet light (364 nm wavelength)).
8. The cyst may be stained with violamine/acridine orange/mix up of light green SF yellowish neutral red and observed under phase-contrast microscope.
9. The further identification of *Azotobacter* at species level includes biochemical tests using specific compounds/conditions in selective medium as mentioned earlier in text and in Table 2 (for *A. paspali*).
10. The *Azotobacter* isolates can be maintained by subculturing at bimonthly interval at Burk's or Winogradsky's medium with sucrose. The agar grown culture can be maintained by suspending in 0.5 ml of 10% (v/v) glycerol or paraffin oil or in 7% dimethyl sulfoxide in 0.1% phosphate buffer (pH 7.0).

4 Notes

1. In N₂-free Winogradsky's medium, the new cells of varying species are appeared enormously analogous and hence the old age culture should be compared for species-level identification.
2. The exopolysaccharide synthesis is the prominent characteristics of the cyst-forming *Azotobacter*.
3. All *Azotobacter* sp. are very susceptible to streptomycin and kanamycin/neomycin.

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Chapter 4

Isolation and Identification of *Azospirillum*

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Abstract

Azospirillum is a free-living nitrogen-fixing member of the α -subclass of proteobacteria. *Azospirillum* is generally found in association with plants and soil and is well known as plant growth promoters. The nitrogen-free semisolid media such as Nfb, RC, FAM, and LGI are employed to isolate *Azospirillum* genus. This chapter includes isolation protocol for *Azospirillum* from plant sample (leaf, stem, and root). Rhizosphere soil and its identification methods are discussed.

Key words *Azospirillum*, Nitrogen fixation, Nitrogen-free semisolid media, 16S rDNA, Molecular identification

1 Introduction

The genus *Azospirillum* was first discovered by Martinus Beijerinck in the year 1925. He named the bacteria initially *Azotobacter largimobile*, later renamed as *Spirillum lipoferum*. After that Johanna Dobreiner identified that the genus *Azospirillum* has the ability to fix atmospheric nitrogen [1] and produce several phytohormones (auxins, cytokinins, and gibberellins) [2]. The *Azospirillum* spp. are Gram-negative, alpha-proteobacteria belongs to the *Rhodospirillaceae* family, and they are versatile in the environment. *Azospirillum* spp. have been reported for the several mechanisms to promote plant growth, such as nitrogen fixation capacity, production of phytohormones, and synthesis of several compounds that help enhance the plant growth against biotic and abiotic stresses [3].

2 Materials

2.1 Isolation of *Azospirillum* spp. from the Soil Sample

1. Soil sample.
2. Dilution tubes.
3. Petri dish.

4. Nfb (nitrogen-free bromothymol blue) semisolid media.
5. Saline solution or sucrose (4%) solution.
6. Rotary shaker.

**2.2 Isolation
of *Azospirillum* spp.
from the Root, Shoot,
and Leaves**

1. Samples (root, shoot, and leaves).
2. 1% chloramine T solution ($\text{CH}_3\cdot\text{C}_6\text{H}_4\cdot\text{SO}_2\cdot\text{NNaCl}\cdot 3\text{H}_2\text{O}$).

**2.3 Identification
of *Azospirillum* spp. by
Biochemical Methods**

1. Refer standard microbiology laboratory manual for biochemical test.

**2.4 Molecular
Identification
of *Azospirillum* spp.**

1. 10% SDS.
2. T₅₀E₂₀ buffer (Tris 50 mM, 20 mM EDTA, pH 8).
3. Pronase E (50 mg/ml).
4. RNase (0.5 µg/µl).
5. Sodium acetate (3 M, pH 5.5).
6. 100% ethanol and 70% ethanol.
7. Gel electrophoresis.
8. PCR fine chemicals as per standard protocol.
9. Primers: *Azo494*-F, 5'-GGC CYG WTY AGT CAG RAG TG-3' and *Azo756*-R, 5'-AAG TGC ATG CAC CCC RRC GTC TAG C-3'.
10. Thermocycler.
11. GelDoc/transilluminator.

3 Methods

**3.1 Isolation
of *Azospirillum* spp.
from the Soil Sample**

1. Collect rhizosphere soil sample using sterile apparatus and keep it in a clean ziplock bag and transfer to the laboratory.
2. Weigh 10 g of soil and homogenize for 30–60 min in 90 ml of saline solution or sucrose solution (4%) under shaker condition (150 rpm).
3. Prepare dilutions (up to 10^{-8}) of the homogenized sample as per standard dilution protocol.
4. Inoculate 0.1 ml of inoculum from the last three dilution tubes (10^{-6} to 10^{-8}) to test tube containing semisolid Nfb medium.
5. Incubate the inoculated tubes into the incubator at 32 °C for 48 h.

6. After the incubation period, observe the growth in the form of pellicles formation.
7. Pick pellicle using wire loop and streak on the Nfb solid media. Incubate the plates at 32 °C for 24 h.
8. Pick morphologically different colony and streak on the basal minimal salt agar medium and incubate at 32 °C for 24 h.
9. Preserve isolated colony for further investigation.

3.2 Isolation of *Azospirillum* spp. from the Root, Shoot, and Leaves

1. Collect plant sample (root, shoot, and leaves) using sterile tools and keep it in the sterile ziplock bag and transfer to the laboratory for the isolation.
2. Surface-sterilize the collected plant sample using 1% chloramine T solution ($\text{CH}_3\cdot\text{C}_6\text{H}_4\cdot\text{SO}_2\cdot\text{NNaCl}\cdot 3\text{H}_2\text{O}$) (see **Note 1**).
3. Wash the samples properly with the sterile distilled water, followed by phosphate buffer (50 mM, pH 7.0), and a final wash with sterile distilled water (repeat this step till chloramine T solution is removed).
4. Weigh 10 g of sterilized plant sample (root, shoot, and leaves) and blend in saline solution or sucrose solution.
5. After 60 min, take an aliquot of 1 ml in 9 ml of saline or sucrose solution to prepare dilution tubes (10^{-8}).
6. Take 0.1 ml sample from each last three dilutions and inoculate in the semisolid media (Nfb), and incubate at 30 °C to 34 °C for 4–7 days.
7. After pellicle formation, transfer into new sterile semisolid Nfb media for 4–7 days. Purify the colony and store it for further investigation.
8. Cultures can be stored at –80 °C for many years or inoculate culture in liquid N_2 with 50% glycerol or dimethylsulfoxide (DMSO).

3.2.1 Media Used for the Isolation of *Azospirillum*

1. *Nitrogen-free semisolid medium (Nfb)*: Add ingredient per liter; D,L-malic acid, 5 g; K_2HPO_4 , 0.5 g; $\text{MgSO}_4\cdot 7\text{H}_2\text{O}$, 0.2 g; NaCl, 0.1 g; $\text{CaCl}_2\cdot 2\text{H}_2\text{O}$, 0.02 g, FeEDTA, solution 1.64%, 4 ml; KOH—control pH, 4.5; bromothymol blue (0.5% in 0.2 N KOH) pH indicator, 2 ml; microelements solution, 2 ml; agar semisolid, 1.75 g; and agar solid media + yeast extract, 17 g.
2. *FAM medium*: Add ingredient per liter; crystal sugar, 5 g; K_2HPO_4 , 0.12 g; KH_2PO_4 , 0.03 g; $\text{MgSO}_4\cdot 7\text{H}_2\text{O}$, 0.2 g; NaCl, 0.1 g; $\text{CaCl}_2\cdot 2\text{H}_2\text{O}$, 0.02 g; $\text{Na}_2\text{MoO}_4\cdot 2\text{H}_2\text{O}$, 0.002 g; FeEDTA, solution 1.64%, 4 ml; final pH, 6.0; agar semisolid, 1.75 g; agar solid media + yeast extract, 15 g.

3. *LGI medium*: Add ingredient per liter; sugar, 5 g; K_2HPO_4 , 0.2 g; KH_2PO_4 , 0.6 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; $CaCl_2 \cdot 2H_2O$, 0.002 g; FeEDTA, solution 1.64%, 4 ml; bromothymol blue (0.5% in 0.2 N KOH) pH indicator, 5 ml; final pH, 6.0; agar semisolid, 1.75 g; agar solid media + yeast extract, 15 g [4].
4. *SM medium*: Add ingredient per liter; D,L-malic acid, 5 g; K_2HPO_4 , 0.13 g; $MgSO_4 \cdot 7H_2O$, 0.25 g; NaCl, 1.2 g; $CaCl_2 \cdot 2H_2O$, 0.22 g; Na_2SO_4 , 2.4 g; $NaHCO_3$, 0.5 g; Na_2CO_3 , 0.09 g; K_2SO_4 , 0.17 g; FeEDTA, solution 1.64%, 4 ml; KOH—control pH, 4.8 g; final pH, 8.5; agar semisolid, 1.75 g; agar solid media + yeast extract, 15 g [4].
5. *M medium*: Add ingredient per liter; D,L-malic acid, 5 g; K_2HPO_4 , 0.4 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; NaCl, 0.1 g; $CaCl_2 \cdot 2H_2O$, 0.02 g; $FeCl_3$, 10 mg; KOH—control pH, 4.8 g; yeast extract, 0.1 g; final pH, 6.8; agar solid media + yeast extract, 15 g.
6. *Rojo Congo (Congo red: RC)*: Add ingredient per liter; KH_2PO_4 , 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; NaCl, 0.1 g; yeast extract, 0.5 g; $FeCl_3 \cdot 6H_2O$, 0.015; D,L-malic acid 5 g; KOH, 4.8 g; and agar 20 g. Adjust pH to 7.0 with 0.1 N KOH. Add 15 ml of 1:400 aqueous solution of Congo red (autoclave separately) [5].

3.2.2 Media Used for Lyophilization Method [6]

K_2HPO_4 , 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; NaCl, 0.1 g; D,L-malate or glucose 5 g; yeast extract, 0.4 g; and distilled water 1 l.

3.3 Identification of Azospirillum (Table 1)

3.3.1 Staining and Biochemical Methods

3.4 Molecular Identification of Azospirillum

1. Refer standard microbiology laboratory manual for biochemical test.
1. Grow culture in suitable media. At late log phase, take 5 ml culture and extract pellet using centrifugation method (at $11200 \times g$ for 10 min).
2. Wash pellet twice with 1.5 ml of $T_{50}E_{20}$ buffer (Tris 50 mM, 20 mM EDTA, pH 8).
3. Lyse cells through adding 7 μ l of pronase E (50 mg/ml), 50 μ l of 10% SDS, and incubate at 37 °C for 1 h.
4. Gently mix up and down the sample using 1 ml syringe to disrupt the DNA physically.
5. Extract the DNA by adding 300 μ l of phenol and 300 μ l of chloroform and mix gently followed by centrifugation at

Table 1
Physiological and biochemical properties of selected *Azospirillum* spp

Tests	<i>A. brasibense</i>	<i>A. lipoferum</i>	<i>A. halopraeferens</i>
Gram staining	—	—	—
Cell	Curved	Curved	Curved
Yeast required for N ₂ -dependent growth	—	—	—
Motility	+	+	+
Oxidase test	+	+	+
Catalase test	+	+	+
Urease test	—	—	—
Nitrate reduction	+	+	+
Starch hydrolysis	—	+	—
Gelatin hydrolysis	—	—	—
<i>Fermentation test</i>			
Glucose	+	+	—
Sucrose	—	—	—
Mannitol	+	—	—
Cellulose	+	+	+
Lactose	+	+	+
Fructose	+	—	+
Dulcitol	+	—	+
Galactose	+	—	—
Xylose	—	—	—
Maltose	+	+	+
Glucose as a sole carbon source	—	+	—
Sucrose as a sole carbon source	—	—	+
Biotin requirement	Not required	Required	Required
Acidification of peptone based on glucose medium	—	+	—
Ammonification	—	+	+
Denitrification	+	+	+

11200 × *g* for 10 min. Repeat the phenol–chloroform step until the supernatant is clear.

6. Add 3 µl of RNase (0.5 µg/µl) and incubate at 37 °C for 30–60 min.
7. Add 1:10 volume of sodium acetate (3 M, pH 5.5) and 2 volume of 100% ethanol.

8. Incubate the tube at -20°C for 2 h or overnight at 4°C .
9. Centrifuge the sample at $11200 \times g$ for 15–20 min at 4°C and collect the DNA.
10. Wash the pellet with chilled 70% ethanol and allow it to dry.
11. Dissolve DNA by adding 200 μl TE buffer and observe the DNA by gel electrophoresis (0.8% agarose mini gel).
12. Perform PCR by using primers: Forward primer *Azo494*-F, 5'-GGC CYG WTY AGT CAG RAG TG-3' and Reverse primer *Azo756*-R, 5'-AAG TGC ATG CAC CCC RRC GTC TAG C-3' [7].
13. Cycling conditions for PCR: Initial denaturation for 2 min at 95°C , 35 cycles of 1 min at 94°C , 1.5 min at 68°C , and 0.5 min at 72°C . Final extension for 7 min at 72°C .
14. Separate amplification products on 1% agarose gel. Compare with standard DNA ladder.

4 Notes

1. For isolation of *Azospirillum*, the surface sterilization varies according to plant type and age. For sorghum and maize at the flowering stage, immerse in disinfectant for 30 min to 1 h. For rice and wheat, immerse for 5–15 min in chloramine T solution.

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Chapter 5

Isolation and Identification of *Gluconacetobacter diazotrophicus*

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Abstract

A symbiotic endophytic diazotroph, *Gluconacetobacter diazotrophicus*, is an efficient nitrogen-fixing bacterium. Colonization of this endophyte is majorly with monocots with special preference in plants having high sucrose concentrations. *G. diazotrophicus* has reported to have plant growth promoting traits and also biocontrol activity. Media supplemented with cane sugar is commonly used for isolation of *G. diazotrophicus* from the plant roots. *G. diazotrophicus* is identified based on morphological, cultural characterization, and biochemical tests.

Key words *G. diazotrophicus*, LGI medium, Diazotroph, Endophyte, Nitrogen fixation

1 Introduction

Gluconacetobacter diazotrophicus (synonymous *Acetobacter diazotrophicus*), a Gram-negative bacteria grouped under the family *Acetobacteraceae* that belongs to the phylum *Proteobacteria*. *G. diazotrophicus* is an acid-producing bacterium that produces acetic acid, hence this bacterium is known to be an acid-tolerant [1]. Initially, it is isolated from plant parts of sugarcane and has also been isolated from grasses like corn, wheat, baby corn, and rice and also from plants such as coffee, pineapple, and carrot [2, 3].

This endophytic bacterium is reported to be located in apoplastic fluid of the crop, especially in which sugar may be free in apoplast [4]. The carbon source that supports the best growth of *G. diazotrophicus* is sucrose at 10%, and it prefers to grow even at high concentrations of sucrose (30%). As this endophyte is unable to transport or respire sucrose, levansucrase, an extracellular enzyme, is secreted for their growth which hydrolyzes sucrose into fructose and glucose [5]. This bacterium is a diazotroph that fixes nitrogen under microaerophilic conditions. More than 50% of the nitrogen requirement of several sugarcane varieties is through

biological nitrogen fixation (BNF). This organism is of special interest as it excretes almost half of the fixed N in a form potentially available to the plants [6].

This endophytic diazotrophic bacterium imparts number of agricultural applications as plant growth promoting rhizobacteria such as nitrogen fixation inside the plants, phosphorus solubilization [7], and production of significant amounts of growth hormone [8] and also as a biocontrol against pathogen [9].

2 Materials

2.1 Isolation of *Gluconacetobacter diazotrophicus*

1. Healthy fresh root.
2. Sterile polycover (sample collection).
3. Distilled water.
4. 0.1% mercuric chloride and 70% ethanol (surface sterilization).
5. N-free LGI semisolid medium (K_2HPO_4 , 0.2 g; KH_2PO_4 , 0.6 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; $CaCl_2 \cdot 2H_2O$, 0.02 g; $Na_2MoO_4 \cdot 2H_2O$, 0.002 g; $FeCl_3 \cdot 6H_2O$, 0.01 g; 0.5% Bromothymol blue in 0.2 N KOH, 5 ml; agar, 2.0 g; crystallized cane sugar, 100 g; and distilled water, 1000 ml; pH —5.5).
6. Glacial acetic acid (pH adjustment).
7. Solid acetic LGI medium (semisolid LGI medium acidified with acetic acid to pH 4.5 and agar concentration increased to 20 g l⁻¹).

2.2 Identification of *Gluconacetobacter diazotrophicus*

2.2.1 Morphological and Biochemical Tests

2.2.2 Cultural Characterization

1. Refer standard microbiology laboratory manual.
1. Potato sucrose agar (peeled potato 200.0 g; sucrose 100.0 g; agar 20.0 g; distilled water 1000 ml; pH 5.5; cook the peeled potatoes for 30 min in 1000 ml of distilled water and use the extract) [9].
2. GYC (glucose–yeast extract– $CaCO_3$) media (glucose 100 g; yeast extract 10 g; calcium carbonate 20 g; and agar 20 g; pH 5.5).
3. YEC (glucose–yeast extract–ethanol– $CaCO_3$) media (yeast extract 10 g; calcium carbonate 20 g; ethanol 30 ml/l; and agar 20 g; pH 5.5).

3 Methods

3.1 Isolation of *Gluconacetobacter diazotrophicus*

1. Uproot the healthy plant and collect the fresh roots. Wash with running tap water.
2. Surface sterilize with 0.1% mercuric chloride for 30 s followed by distilled water wash and then with 70% alcohol for 1 min and immediately wash with distilled water for 3–4 times repeatedly.
3. Weigh the sample and homogenize it with sterile sucrose solution (1%) using pestle and mortar.
4. Inoculate the tubes containing semisolid LGI medium using 0.5 ml of aliquot.
5. Incubate at $28^{\circ} \pm 2^{\circ}\text{C}$ without disturbance for 6–7 days. Observe for formation of orange-yellow subsurface pellicles in the tube.
6. Streak the orange to yellowish bacterial growth onto the LGI agar plates.
7. Incubate for 7 days at $28^{\circ}\text{C} \pm 2^{\circ}\text{C}$. After incubation, select the orange colonies and purify as per standard procedure.
8. Store the pure culture of the strain as a glycerol stock at -80°C for further studies.

3.2 Identification of *Gluconacetobacter diazotrophicus*

3.2.1 Morphological Characterization

1. Perform the staining using Gram-staining technique as per standard protocol.
2. Observe the motility by carrying out stabbing method in semisolid medium as per standard procedure.
3. Infer the results with already existing result of *Gluconacetobacter diazotrophicus* (Table 1).

3.2.2 Cultural Characterization

1. Inoculate the fresh culture in the following media [9, 10].
2. N-free semisolid LGI medium.
3. Potato sucrose agar.
4. GYC agar medium.
5. YEC agar medium.
6. 30% sucrose in semisolid LGI tubes.
7. Incubate at $28^{\circ}\text{C} \pm 2^{\circ}\text{C}$ for 7–10 days. Observe the cultural characteristics and infer the results with already existing results (Table 1).

Table 1
Characteristics of isolated *Gluconacetobacter diazotrophicus* [11–13]

Characteristics	Results
Gram staining	Negative
Motility	Positive
Endospore	Negative
N-free semisolid LGI medium at pH 4.5	Yellow subsurface pellicle formation
Potato sucrose agar	Brown colonies
GYC agar medium	Over oxidation of the glucose and formation of water-soluble brown pigments
YEC agar medium	Precipitation of CaCO_3 and over oxidation of ethanol
Growth in 30% sucrose	Positive
Catalase	Positive
Oxidase	Negative
Gelatin hydrolysis	Negative
Starch hydrolysis	Negative
Cellulase activity	Negative
Formation of H_2S	Negative
Nitrate reduction	Negative

3.2.3 Biochemical Characterization

1. Carry out biochemical tests according to the standard protocol.
2. Compare and conclude the results with previously prevailing outcomes (Table 1).

4 Notes

1. Adjust the pH of the media using glacial acetic acid.
2. Add cycloheximide to the media to avoid fungal growth.
3. Use freshly prepared media for better results.

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Chapter 6

Isolation and Identification of Nitrogen Fixing Bacteria: *Azoarcus* Species

Rashmi Thakor, Harsh Mistry, and Himanshu Bariya 

Abstract

Nitrogen is the most important element for all the organisms. It is beneficial for the growth and development of plants. Biological nitrogen fixation is found to be an efficient approach for the availability of nitrogen to the plants using diazotrophic bacteria such as *Azoarcus* species. The present chapter focuses on the methods for the isolation of *Azoarcus* species on different media and its identification using 16S rDNA sequencing with specific primers for *Azoarcus*, as the potent nitrogen fixing bacteria from different sources.

Key words *Azoarcus* spp., Isolation, Identification, Characterization, 16S rDNA sequencing, Nitrogen fixing bacteria

1 Introduction

Nitrogen is an indispensable element of life which plays a significant role in building proteins and DNA. Despite of being available in massive amounts in the atmosphere, limited quantity of soil inorganic nitrogen is attainable to plants in the form of ammonium and nitrate, often limiting agricultural yields by nitrogen availability. Thus, biological nitrogen fixation seems to be an effective approach for providing nitrogen to crops and plants. For the sustainable agricultural production and better functioning of ecosystem, biological nitrogen fixation in plants seems to be an important mechanism. Biological nitrogen fixation is carried out by bacteria, especially rhizobacteria, archaea, prokaryotes such as diazotrophs (free-living or present in symbiosis), etc. Among diazotrophs, *Azoarcus* was found to be an efficient colonizer and sequenced genome [1, 2].

Azoarcus, genus of Gram-negative bacteria, is classified among the family *Rhodocyclaceae* of the order *Rhodocyclales* in the class *Betaproteobacteria*. The fatty acid profile of the members of *Azoarcus* indicates C_{16:0}, C_{16:1}, and C_{18:1} as the considerate fatty acid and

the DNA G+C contents within the range of 62.4–67.8 mol%. They also contain poly- β -hydroxybutyrate (PHB) granules. These PHB granules store carbon and energy in numerous prokaryotes, which facilitates microbe's survival in the absence of carbon sources. They are chemoorganoheterotrophic and oxidase positive in nature. They are broadly distributed in diverse environments. Except *Azoarcus anaerobius*, the stringent respiratory metabolism with O₂ as the terminal electron acceptor has been observed in the members of this genus. Nitrogen is fixed by some of the species, but then microaerobic conditions are required for their growth on N₂. Some of the recognized species of the genus *Azoarcus* are *Azoarcus communis*, *Azoarcus indigenus*, *Azoarcus toluyticus*, *Azoarcus anaerobius*, *Azoarcus evansii*, *Azoarcus toluvorans*, *Azoarcus buckelii*, *Azoarcus nasutitermitis*, *Azoarcus rhizosphaerae*, and *Azoarcus toluclasticus* [3–6]. The present chapter focuses on the isolation and identification of nitrogen fixing bacteria, particularly *Azoarcus* species.

2 Materials

2.1 Media for *Azoarcus* Isolation

Azoarcus is the genus of Gram-negative bacteria, firstly described in 1993 and belongs to the *Rhodocyclaceae* family. It was firstly described as a nitrogen fixing endophytes and found in grass, sea water, oxic soil, hot springs, petroleum contaminated sites, and roots and stems of plants. The compositions of the media are given below and can be used for the isolation of *Azoarcus*. The media must be autoclaved at 121 °C and 15 psi for 15 min, cool down to 50 °C, and pour into the petri dishes. Plate pouring and other necessary work should be performed in laminar air flow, and the work area should be sterilized with 70% ethanol prior to work to prevent microbial contamination (Tables 1, 2 and 3).

2.2 Isolation of *Azoarcus* from Soil

1. Rhizospheric soil sample or contaminated soil sample.
2. Sterilized sample collection containers.
3. Conical flasks (several volumes).
4. Sterile distilled water.
5. Sterile petri plates.
6. 70% ethanol.
7. Glass spreaders.
8. Disposable gloves.
9. Lab coat.

Table 1
Composition of nutrient agar

Sr. No.	Compound	Formula	Concentration (g/L)
1	Peptone		5.0
2	Sodium chloride	NaCl	5.0
3	Beef extract		1.5
4	Yeast extract		1.5
5	Agar		15.0

Table 2
Composition of nitrogen-free solid media

Sr. No.	Compound	Formula	Concentration (g/L)
1	Sucrose	$C_{12}H_{22}O_{11}$	20.0
2	Sodium chloride	NaCl	0.2
3	Dipotassium phosphate	K_2HPO_4	0.2
4	Magnesium sulfate	$MgSO_4 \cdot 7H_2O$	0.2
5	Potassium sulfate	K_2SO_4	0.1
6	Calcium carbonate	$CaCO_3$	5.0
7	Agar	—	20.0

Table 3
Composition of Congo red–yeast extract mannitol agar (CR-YEMA)

Sr. No.	Compound	Formula	Concentration (g/L)
1	Yeast extract	—	0.4
2	Mannitol	$C_6H_{14}O_6$	10.0
3	Dipotassium phosphate	K_2HPO_4	0.5
4	Magnesium sulphate	$MgSO_4 \cdot 7H_2O$	0.2
5	Sodium chloride	NaCl	0.1
6	Congo red	$C_{32}H_{22}N_6Na_2O_6S_2$	0.025
7	Agar	—	15

10. Wire loop.
11. Shaker.
12. Autoclave.
13. Incubator.

2.3 Isolation of Azoarcus from Plant Samples

1. Plant sample.
2. Sterile scalpel.
3. Watch glass.
4. Sodium hypochlorite.
5. 6% H₂O₂ solution.
6. 1% glutaraldehyde.
7. Congo red.
8. Vials.
9. Spade.
10. Tea strainer bag or muslin cloth.
11. Activated silica beads.
12. Other materials as mentioned in Subheading [2.2](#).

2.4 Gram Staining

1. Slide.
2. Coverslip.
3. Distilled water.
4. HiMedia Grams Stain-Kit K001.
5. Microscope.

2.5 Molecular Identification (DNA Isolation, Amplification, and Sequencing)

1. 0.8% agarose gel for genomic DNA.
 - (a) Add 0.8 g agarose powder to 100 mL 1 × TAE buffer.
 - (b) Sprinkle agarose powder. Heat the flask until it gets digested.
 - (c) Cool down until it is not painful to touch and add 3 µL of ethidium bromide.
 - (d) Cast the gel in casting assembly and allow it to get solidify.
2. 1.2% agarose gel for PCR amplified DNA.
 - (a) Add 1.2 g agarose powder to 100 mL 1 × TAE buffer.
 - (b) Follow the steps from **2** to **4** abovementioned.
3. Centrifuge.
4. Agarose gel electrophoresis apparatus.
5. UV transilluminator.
6. PCR.
7. Primers (forward and reverse).
8. DNA sequencers and analyzers.
9. Micropipette.
10. HiPer[®] Bacterial Genomic DNA Extraction Teaching Kit HTBM008.

11. GenElute™ PCR Clean-up kit.
12. BigDye® Terminator v 3.1 Cycle sequencing kit.

3 Method

3.1 Isolation of Nitrogen Fixing Bacteria from Soil Sample

1. Soil samples such as rhizospheric soil and contaminated soils can be taken from the depth of 15–20 cm, for the isolation of nitrogen fixing bacteria.
2. Use sterile spatula to collect soil samples in a sterile sample collection container.
3. Bring the collected sample immediately to the laboratory and store at 4 °C until the further use.
4. Prepare stock solution by adding 1 g of soil sample in 100 mL of sterile distilled water in a conical flask. Shake it at 120 rpm for 30 min to 1 h.
5. Add 1 mL stock mixture in 9 mL of sterile distilled water in a conical flask to acquire 10^{-1} soil suspension.
6. Use sterile distilled water to obtain 10^{-2} , 10^{-3} , 10^{-4} , and 10^{-5} dilutions of soil suspension.
7. Inoculate 100 µL soil suspensions of each dilution into the sterile petri plates containing agar media (nutrient and N-free agar).
8. Spread the inoculum with the help of the sterile glass spreader.
9. Incubate all the plates at 28 ± 1 °C in BOD incubator for 5–7 days.
10. Transfer bacterial colonies onto fresh nutrient agar plates to obtain a pure culture.

3.2 Isolation of Nitrogen Fixing Bacteria from Plant Root Nodule Sample

1. Dig out healthy nodules bearing plants using spade in the form of a circle with the approximate radius of about 10 cm and 30 cm depth around the plant from the field.
2. Sieve the excavated plants and flush gently with water to wash out all the adherent soil particles.
3. Record the data related to number of nodules collected per plants along with the location of the sampling sites.
4. For isolation of rhizobia, collect 10–15 nodules per plant. Air-dry the nodules and store it in the vials containing activated silica beads. Some nodules are fixed in vials comprising 1% glutaraldehyde for microscopic studies.
5. Wash out the dug root nodules properly using sterile distilled water. Soak the dried nodules in sterile distilled water for 3–4 h to rehydrate them if stored in activated silica beads.

6. Place nodules in tea strainers or tie them in a muslin cloth and perform surface sterilization to avoid epiphytes or other microbial contamination by keeping nodules in 70% ethanol for 30 s followed by sterile distilled water for 30 s. Then, transfer the washed nodules in 1% sodium hypochlorite or 6% H₂O₂ solution for 30 s and rinse with sterile distilled water.
7. Place nodules in a watch glass and cut into 2 halves using sterile scalpel. Add a drop of sterile distilled water to section. Streak white exudate from the section on CR-YEM agar plates and incubate at 28 °C.

4 Identification

4.1 General Morphology of *Azoarcus* spp. on the Solid Agar Media

1. The genus *Azoarcus* contains rod-shaped Gram-negative bacteria.
2. Presence of flagella is observed.
3. They are positive for catalase and oxidase activities.
4. They are negative for DNase and proteinase reactions.
5. Mostly they are aerobic bacteria.

4.2 Characteristics of *Azoarcus* Strains

Sr No.	Tests	<i>Azoarcus</i> spp. strain BH72	<i>Azoarcus indigenus</i>	<i>Azoarcus communis</i>	<i>Azoarcus</i> spp. strains 6a3, 6a2, and 5c1
1.	Gram staining	—	—	—	—
2.	Motility	+	+	+	+
3.	Catalase	+	+	+	+
4.	Nitrate reduction	+	+	—	—
5.	Voges-Proskauer	—	+	—	+
6.	Urease	—	+	—	—
7.	Sole carbon sources used for growth				
a.	L-Aspartate	+	+	—	+
b.	D-Malate	+	+	+	—
c.	Glutarate	+	—	+	—
d.	L-Proline	—	—	—	—
e.	Phenylacetate	+	+	±	—
8.	DNA G+C content (mol%)	67.6	66.6	62.4	65.2

4.3 Molecular Identification

Accurate identification of *Azoarcus* at species level implies the use of molecular methods, precisely gene sequencing and analysis. Molecular identification of *Azoarcus* involves following steps:

1. Isolation of genomic DNA from the sample.
2. Amplification of the DNA fragment using high-fidelity PCR polymerase.
3. Sequencing of the PCR product bi-directionally.
4. Alignment analysis of the sequenced data to identify their closest neighbor genetics.

4.3.1 Isolation of Genomic DNA

Perform isolation of genomic DNA as per instruction given by HiPer[®] Bacterial Genomic DNA Extraction Teaching Kit and run 0.8% agarose gel for genomic DNA.

4.3.2 Amplification of the DNA Fragment

Amplification of DNA fragments involves following steps:

1. Prepare the PCR mixture as described in PCR mixture.
2. Set the PCR amplification conditions as described in PCR amplification condition.
3. Check PCR product on 1.2% agarose gel.

PCR Mixture

Sr. No	Reactants	Quantity
1	DNA	1 µL
2	Forward primer	400 ng
3	Reverse primer	400 ng
4	dNTPs (2.5 mM each)	4 µL
5	10x TaqDNA polymerase assay buffer	10 µL
6	TaqDNA polymerase (3 U/mL)	1 µL
7	Water	µL
Total reaction volume		100 µL

PCR Amplification Condition

Sr. No	Reactions	Temperature (°C)	Time	Cycle
1	Initial denaturation	98	5 min	1
2	Denaturation	98	15 s	25
3	Annealing	62	30 s	
4	Synthesis	72	10 s	
5	Extension	72	5 min	1

Primers

Sr. No	Primer name	Direction	Primer sequence (5'-3')	Organism	References
1	8F	Forward	AGAGTTTGATC CTGGCTCAG	Bacteria (Most common universal primers)	[7]
	27F	Forward	AGAGTTTGATC MTGGCTCAG		
	U1492R	Reverse	GGTACCTTG TTACGACTT		
2	TH3	Forward	GATTGGAGCG GCCGATGTC	<i>Azoarcus spp.</i> (specific primers)	[8]
	TH5	Reverse	CTGGTTCCCG AAGGCACCC		
3	TH14	Forward	GCTAATACCGC ATACGTCCT GAGGG		
	TH2	Reverse	AACGCTCGCAC CCTCGTATTA CCGC		

4.3.3 Sequencing of the PCR Product

Carry out the purification of gene amplified products using a GenElute™ PCR Clean-up kit (cat no. NA 1020-1kt).

Purification of Amplified PCR Products

Cycle Sequencing

Use BigDye® Terminator v 3.1 Cycle sequencing kit for cycle sequencing. The BigDye Terminator v3.1 Cycle sequencing kit provides the required reagent components for the sequencing reaction in a ready reaction, premixed format.

Cycle Sequencing Reagent and Concentration

Sr. No	Reagents	Quantity
1	Terminator ready reaction mix v3.1	4 µL
2	BigDye sequencing buffer	1 µL
3	Template	15–300 ng
4	Primer	10 pM
5	Milli-Q water	Make up to 20 µL

Cycle Sequencing
Conditions

Sr. No	Reactions	Temperature (°C)	Time	Cycle
1	Incubation	96	1 min	—
2	Denaturation	96	10 s	25
3	Annealing	50	5 s	
4	Extension	60	4 min	
5	Hold	4	∞	—

Capillary Electrophoresis
Parameters

Polymer	Array	Run module	Mobility file
POP-7™ polymer	50 cm	BDX_StdSeq50_ POP7xl	KB_3500_POP7xl_BDTv3direct. mob

4.3.4 Alignment Analysis
of the Sequenced Data to
Identify Their Closest
Neighbor Genetics

1. Submit raw data obtained from sequencing to NCBI to obtain accession number.
2. Download the FASTA format from NCBI.
3. Process data in MEGA X to construct phylogenetic tree by using maximum likelihood method and Jukes-Cantor model.

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Chapter 7

Isolation and Identification of *Derxia* Species from the Soil Sample

Harshida A. Gamit and Natarajan Amaresan

Abstract

Derxia is a nitrogen-fixing bacteria under aerobic or microaerobic conditions. In genus *Derxia*, only two species are recognized: *Derxia gummosa* isolated from a soil sample and *Derxia lacustris* isolated from freshwater. Generally, isolation of *Derxia* done by using sieved soil method on nitrogen-free mannitol agar medium. *Derxia* is quite different from the genera *Azomonas*, *Azotobacter*, and *Beijerinckia* using rRNA cistron similarity analysis.

Key words *Derxia*, Sieved soil method, N₂ fixation, Mannitol agar

1 Introduction

Genus *Derxia* was first recognized by Jennsen et al. [1] from Indian soil; *Derxia* belongs to the family *Alcaligenaceae* of the order *Burkholderiales* and class *Betaproteobacteria*. Genus *Derxia* consists of single species *Derxia gummosa*, which is usually found in the tropical area. *Derxia* cells are Gram-negative, rod-shaped with round ends, 1.0–1.2 µm in width, oxidative positive, catalase-negative, motile through short polar flagellum, pleomorphic depending on age and growth medium. *Derxia* strain is acid-tolerant and has been isolated using liquid acidic nitrogen-free glucose medium from culture designed to enrich *Beijerinckia* [2–4]. The genus *Derxia* considered to have a relationship with the diazotrophic genera, *Beijerinckia*, *Pseudomonas*, *Azotobacter*, and *Azomonas* based on physiological, morphological, and chemotaxonomic characteristics; based on the method of rRNA cistron similarity they are quite different from each other. The *Derxia* is extremely sensitive to chloramphenicol, aureomycin, streptomycin, tetracycline, penicillin, and sensitive to polymyxin and oleandomycin [5]. Nitrogen fixation capacity of *D. gummosa* differs between

9 and 25 mg N/g of glucose consumed, but significantly smaller than the *Azotobacter* or *Beijerinckia*.

2 Materials

2.1 Isolation of *Derxia* from Soil Sample

1. Soil sample.
2. Sieve.
3. Nitrogen free-mannitol media.
4. Petri plates.
5. Incubator.

2.2 Staining and Biochemical Methods

1. Refer standard microbiology laboratory manual.

2.3 Molecular Identification [6]

1. PCR fine chemicals as per standard protocols.
2. Primers 520F: 5- CAGCAGCCGCGGTAATAC-3, 1100R: 5- GGGTTGCGCTCGTTTG -3; 926F: 5- AAAC TCAAAG GAATTGACGG-3, 1510R: 5-GGCTACCTTGTTACGTA-3; 8F: 5-AGAGTTTGATCCTGGCTCAG-3, 700R: 5-TCTACG CATTTCACC-3.
3. Thermocycler.
4. GelDoc/transilluminator.

3 Methods [5]

3.1 Isolation of *Derxia* from Soil Sample

1. Use the sieved soil method for the isolation of *Derxia*.
2. Sieve the collected soil sample and evenly distribute over the surface of the nitrogen-free mannitol medium. The following media can be used for the isolation of *Derxia*:
 - (a) **IAM B-1 medium** (Beef extract, 3.0 g; peptone, 5.0 g; NaCl; pH 7.0; distilled water, 1 l) [6].
 - (b) **LMG medium 10** (Glucose, 10.0 g; CaCl₂·2H₂O, 0.1 g; MgSO₄·7H₂O, 0.1 g; K₂HPO₄, 0.9 g; FeSO₄·7H₂O, 10.0 mg; KH₂PO₄, 0.1 g; CaCO₃, 5.0 g; Na₂MoO₄·2H₂O, 5.0 mg; pH: 7.3; distilled water, 1 l) [6].
 - (c) ***Derxia* isolation medium** (Starch, 20.0 g; K₂HPO₄, 0.05 g; MgSO₄·7H₂O, 0.15 g; CaCl₂, 0.02 g; NaHCO₃, 0.1 g; FeCl₃ (10%, aqueous solution), 1 drop; Na₂MoO₄·2H₂O, 0.002 g; Bromothymol blue (0.5%, ethanol solution agar), 5 ml; agar, 20.0 g; distilled water, 1 l) [2].

3. Incubate the plates at room temperature for 4–5 days.
4. After incubation period observe the plates. (Yellowish colonies around the soil particles gradually increase in size and finally assumed in rust-brown).
5. Purify the colony through repeating streaking on nitrogen-free agar medium.
6. Prepare the stock culture to maintain a nutrient-free agar medium and test periodically. The following media can be used for the maintenance of *Derxia*:
 - (a) **Nitrogen-free, mineral glucose agar medium** (Glucose, 10.0 g; K_2HPO_4 , 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.25 g; NaCl, 0.25 g; $FeSO_4 \cdot 7H_2O$, 0.1 g; $CaCl_2$ or $CaCO_3$, 0.1 g; $Na_2MoO_4 \cdot 2H_2O$, 0.005 g; agar, 15.0 g; pH: 6.9; distilled water, 1 l).
 - (b) **Nitrogen-free, mineral starch agar medium** (Starch, 0.05 g; K_2HPO_4 , 0.15 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; $CaCl_2$, 0.02 g; $NaHCO_3$, 0.1 g; $FeCl_3$ solution (10% aqueous solution), 1 drop; $Na_2MoO_4 \cdot 2H_2O$, 0.002 g; Bromothymol blue solution (0.5% ethanol solution), 5.0 ml; agar, 20.0 g; distilled water, 1 l) [2].
 - (c) **Nitrogen-free, mineral mannitol agar medium** (Mannitol, 10.0 g; K_2HPO_4 , 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; $CaCl_2$, 0.1 g; $CaCO_3$, 5.0 g; $Na_2WO_4 \cdot 2H_2O$, 0.0005 g; $FeCl_3$ and $Na_2MoO_4 \cdot 2H_2O$, trace amount; agar, 15.0 g; distilled water, 1 l) [5].
7. The *Derxia* can be preserved or store at +4 °C for 25 months and at –4 °C for 20 months.

3.2 Identification of *Derxia*

1. Perform the staining technique and other biochemical tests as per standard procedures.
2. Infer the results with already available results of *Derxia* (Table 1).

3.3 Molecular Identification

1. Extract the genomic DNA from *Derxia* as per standard protocol using phenol–chloroform extraction method.
2. Perform PCR amplifications in a total volume of 50 µl by mixing 20 ng of the template DNA with 2.5 mM concentrations of each deoxynucleotide triphosphate, 1 µM concentrations of any primer sets given in Subheading 3, and 0.3 U of *Taq* DNA polymerase in 10× *Taq* buffer.
3. Amplification reaction condition includes initial denaturation of 92 °C for 2 min followed by 30 cycles of 92 °C for 1 min, 55 °C for 1 min, 72 °C for 2 min, and then final extension at 72 °C for 5 min.

Table 1
Characteristics of *Derxia gummosa*

<i>Physiological characteristics of D. gummosa</i>			
Characteristics	Reaction	Characteristics	Reaction
Motility	+	Urea hydrolyzed	+
Colony color after aging	Brown	H ₂ S production from cysteine	—
Resistant to 1% peptone	+	Tween	20 hydrolyzed
+			
Starch hydrolyzed	—	Indole produced	+
Growth on asparagine as C and N source	+	Antagonism to gram-positive organisms	+
<i>Morphological characteristics of D. gummosa</i>			
Cell-shaped	Rod-shaped with round ends	Optimum pH	5.5
Cell size	1.0–1.2 µm × 3.0–6.0 µm	Growth in broth medium	Culture turns into a gelatinous mass
Cytoplasm	Young cell-homogeneous cytoplasm	Colonies on agar medium	At first slimy semitransparent, later massive and opaque, highly raised with a wrinkled surface, the color gradually becomes dark brown
Motility	Motile short polar flagellum (rare on nitrogen deficient solid media)	Catalase activity	Negative
N ₂ fixation	Under aerobic or micro aerobic conditions	Gram staining	Gram-negative
Optimum temperature	25 °C to 35 °C	GC content of the DNA	69.2–72.6%
<i>Utilization of carbon compounds by D. gummosa</i>			
Arabinose	—	Oxaloacetate	+
Galactose	—	Fumarate	+
Fructose	+	Malate	—
Melibiose	—	Malonate	+
Maltose	—	Glycolate	+

(continued)

Table 1
(continued)

<i>Physiological characteristics of D. gummosa</i>			
Characteristics	Reaction	Characteristics	Reaction
Mannose	—	Benzoate	—
Sorbose	—	L-ascorbate	—
Raffinose	—	Aspartate	+
Xylose	—	Glutamate	+
Butanol	+	Ethylamine	+
Propanol	+	Mannitol	D
Glycerol	+	Acetate	+
Sorbitol	D	Citrate	+

4. Perform gel electrophoresis to check the amplified product along with desired DNA markers.

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Chapter 8

Isolation and Characterization of *Enterobacter*, *Klebsiella*, and *Clostridium*

V. Mageshwaran and K. Pandiyan

Abstract

The free-living nitrogen fixing bacteria (*Enterobacter*, *Klebsiella*, and *Clostridium*) play significant role in fixation of atmospheric nitrogen and nitrogen economy in crop cultivation. *Enterobacter* and *Klebsiella* are facultative anaerobes and Gram-negative bacteria, whereas *Clostridium* is strict anaerobe and Gram-positive bacteria. These bacteria inhabit soil as well as within the plants and do their function of nitrogen fixation. Thus, these bacteria could be isolated from soil and plant parts as endophytes. The priming of these microbes for supplementation of nitrogen to the crop plants and improvement of crop yield is well documented in earlier reports. In the present chapter, the methods involved in isolation of *Enterobacter*, *Klebsiella*, and *Clostridium* from soil as well as plant parts have been discussed.

Key words *Enterobacter*, *Klebsiella*, *Clostridium*, Isolation, Characterization, Nitrogen fixation

1 Introduction

Nitrogen cycle is the transformation of nitrogen compound from one form to another, and it is an important natural phenomenon for existence of living organisms in the earth. The major groups of microorganisms, bacteria (aerobic and anaerobic), fungi, and actinomycetes play key role in nitrogen cycle in the environment. Nitrogen fixation plays a significant role in ecological balance of nitrogen and nitrogen economy in crop cultivation. Nitrogen fixation takes place by biological and nonbiological while the former predominantly contributes than the later. Annually, about 175 and 80 million metric tons of nitrogen fixed globally by biological and chemical nitrogen fixation process, respectively [1]. Biological nitrogen fixation is environmental friendly and less energy intensive process compared to chemical nitrogen fixation (Haber-Bosch process) in supplying nitrogen for sustainable crop production.

The symbiotic nitrogen fixation in legume–rhizobium symbiosis plays dominant role in nitrogen economy in crop production. At

the same time, the contribution of free-living and associative nitrogen fixing bacteria in fixation of nitrogen in soil is also well documented and acknowledged. The bacterial species, *Herbaspirillum*, *Azospirillum*, *Gluconobacter*, *Enterobacter*, *Klebsiella*, and anaerobic bacterium, *Clostridium* sp., are the predominant free-living nitrogen fixing bacteria especially under anoxic conditions like wetland rice ecosystem [2–4]. *Clostridium* is Gram-positive, strictly anaerobic nitrogen fixing bacteria while *Enterobacter* and *Klebsiella* are Gram-negative, facultative anaerobic nitrogen fixing bacteria. In Bergey's manual of systematic bacteriology (Vol. II, second edition), *Klebsiella* and *Enterobacter* are placed in phylum gamma-proteobacteria while *Clostridium* is placed in phylum firmicutes [5]. In this chapter, the methods involved in isolation and characterization of free-living nitrogen fixers (*Enterobacter*, *Klebsiella*, and *Clostridium*) have been discussed.

2 Materials

2.1 Isolation of *Enterobacter*, *Klebsiella*, and *Clostridium*

1. Scoop for soil sample collection.
2. Sterile polybags for storage of samples.
3. Spatula.
4. Weighing boats.
5. 9 ml sterile saline blanks (0.85% NaCl).
6. Sterile petri plates.
7. L-spreader.
8. Inoculation loop.
9. Bunsen burner.
10. Sterile pestle and mortar.
11. Sterile muslin cloth.
12. Sterile distilled water (for wash).
13. 70% ethanol.
14. 2% sodium hypochlorite.
15. Weighing machine.
16. Laminar air-flow chamber.
17. Autoclave.
18. BOD incubator.
19. Anaeropack/gaspack (for isolation of *Clostridium*).
20. Low nitrogen/nitrogen-free semisolid agar medium (glucose–yeast extract (GYE), Jensen, RMR [3], and VL medium). The composition of the media is given in Table 1.

Table 1**Composition of different semisolid medium used for isolation of nitrogen fixing *Enterobacter*, *Klebsiella*, and *Clostridium***

G-YE medium [2] (g/l)	V L medium [7] (g/l)	Jensen medium ^a (g/l)
Glucose ^b : 10	Nutrient broth: 0.8	Sucrose: 20
Yeast extract: 0.1	Yeast extract: 5	Dipotassium phosphate: 0.5
Agar: 2	NaCl: 5	Magnesium sulphate: 0.5
	Glucose: 2	Sodium chloride: 0.5
	Cysteine-HCl: 0.3	Ferrous sulphate: 0.005
	Agar: 2	Calcium carbonate: 2
		Agar: 2

^aRefer HiMedia Technical data M973^bSeparately autoclave glucose and add to the medium

2.2 Morphological and Biochemical Characterization

1. Procure staining and biochemical kits for bacterial characterization from any standard chemical supplier.

2.3 Molecular Identification

1. Procure DNA isolation kits and PCR chemicals from any reputed chemical supplier.
2. Procure Universal primer pairs [6] MR forward (19 mer [5'-GAG TTT GAT CMT GGC TCA G-3']) and MR reverse (18 mer [5'-ACG GYT ACC TTG TTA CGA CTT-3']).
3. Thermocycler.
4. GelDoc/transilluminator.

3 Methods

3.1 Isolation of *Enterobacter*, *Klebsiella*, and *Clostridium*

1. Collect the soil samples at 0–15 cm depth with the help of scoop and keep the collected samples in sterile polybags.
2. In case of plant samples, uproot the whole plant and keep in sterile polybags.

3.1.1 Collection of Samples

3.1.2 Sample Preparation for Endophytes Isolation

1. Remove the debris and soil particles from the plant parts (roots/stem/leaf) with ample of running tap water.
2. Surface-sterilize the plant parts with the help of 70% ethanol for 1 min followed by rinse with sterile distilled water.
3. Again, surface-sterilize with 2% sodium hypochlorite for 5 min followed by rinse with sterile distilled water.
4. Macerate the surface-sterilized plant parts in sterile pestle and mortar containing sterile water.

5. Pass the macerated sample in sterile muslin cloth and use the aliquot obtained for isolation of endophytic free-living nitrogen fixing bacteria in a suitable medium.

3.1.3 Isolation and Screening

1. Take 1 g of soil/1 ml of aliquot (in case of plant sample) and add in 9 ml of sterile saline blanks and prepare serial dilutions up to 10^{-4} .
2. Add 1 ml of each dilution (10^{-1} to 10^{-4}) in N-free or N-low semisolid media, Jensens and G-YE medium (as given in Subheading 2.1 and Table 1) prepared in test tubes. A minimum of five replications has to be kept for each dilution.
3. Incubate the tubes at 30 °C in a BOD incubator for 1 week period.
4. To detect and screen the nitrogen fixing ability, conduct acetylene reduction assay (ARA) in all test tubes after the incubation period [3].
5. Spread ARA-positive cultures using sterile L-spreader in Jensen/G-YE agar medium and keep for incubation at 30 °C for 2 days. For agar medium, use 15–20 g/l of agar in the medium.
6. Repeat the subculture till the pure culture is obtained. The outline of protocol of isolation and screening of free-living nitrogen fixing bacteria, *Klebsiella*, *Enterobacter*, and *Clostridium* is given in Fig. 1.

Note:: For isolation of anaerobic nitrogen fixing bacteria, *Clostridium* sp., in this case, preheat ARA-positive cultures at 70 °C for 10 min and treat with 50% ethanol for 45 min. Spread the pre-treated cultures using sterile L-spreader in VL/RMR medium agar medium (as given in Subheading 2.1 and Table 1) and keep for incubation under anaerobic condition at 30 °C for 3 days using anaeropack/gaspack system [7].

3.2 Morphological and Biochemical Characterization

1. Perform morphological and biochemical characterization of isolates by referring any standard microbiology laboratory manual.
2. The important morphological characteristics are colony characteristics, Gram staining, endospore staining, and motility, and the major biochemical characteristics are citrate utilization, urease activity, catalase, oxidase, gelatin hydrolysis, indole production, methyl-red test, and Voges–Proskauer test.
3. Infer the results with morphological and biochemical characteristics of *Enterobacter* and *Klebsiella* [2, 5, 8] as given in Tables 2 and 3, respectively.

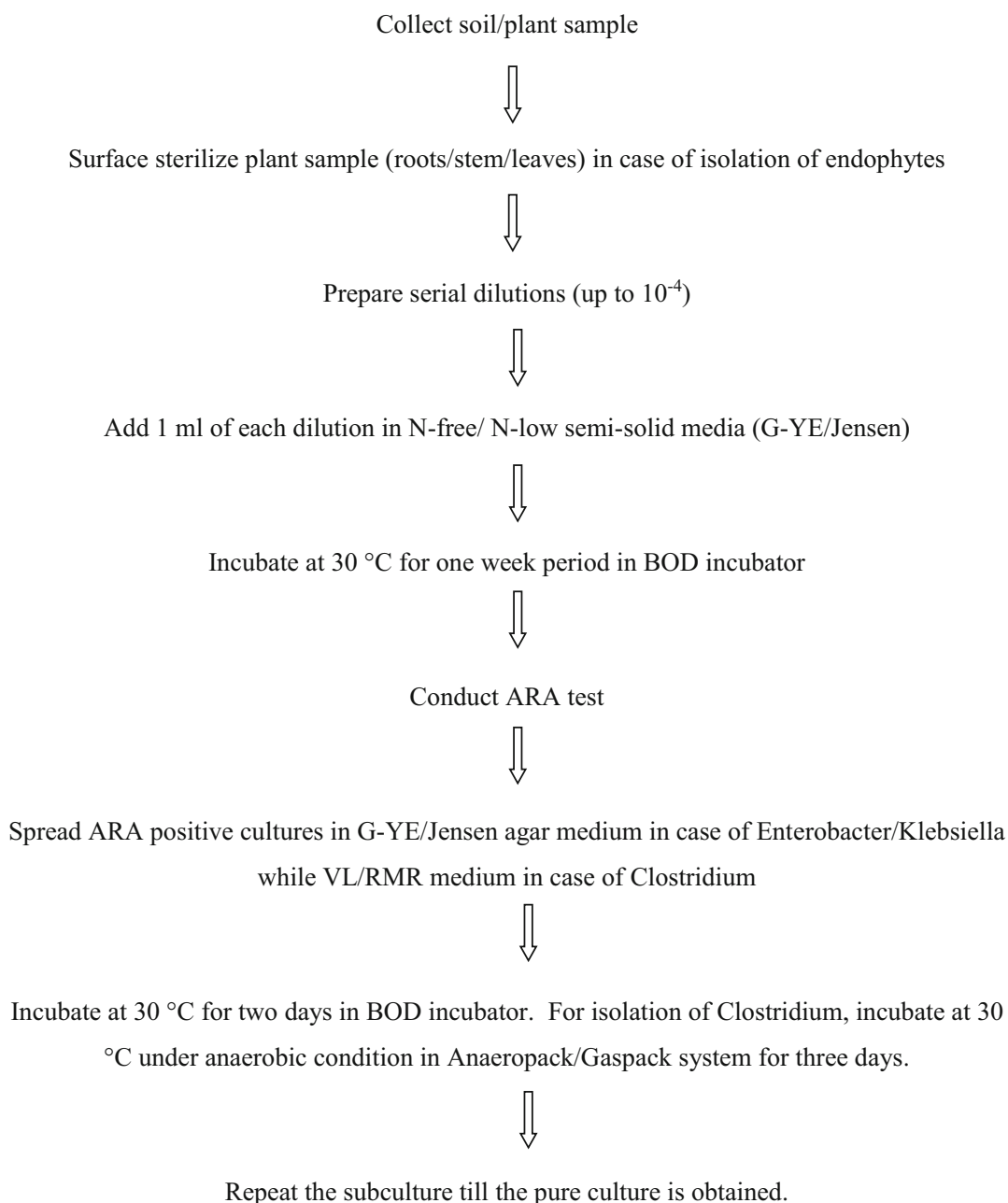


Fig. 1 Outline of isolation of free-living nitrogen fixing bacteria (*Enterobacter*, *Klebsiella*, and *Clostridium*)

4. Infer the results with morphological and biochemical characteristics of *Clostridium* [9] as given in Table 4. The important characteristics of *Clostridium* are Gram-positive, rod-shaped terminal endospore-forming bacteria, produce acetate and propionate in the medium.

Table 2
Differential characteristics of selected nitrogen fixing *Enterobacter* [2, 5, 8]

Characteristics	Enterobacter	
	<i>E. cloacae</i>	<i>E. kobei</i>
Gram stain	—	—
Motility	+	+
Acid and gas production in glucose	+	+
Gelatin hydrolysis	+	—
Methyl-red	—	—
Voges–Proskauer	+	+
Indole production	—	—
Citrate utilization	+	+
Urease	+	+
Catalase	+	+
Oxidase	—	—

(–) negative; (+) positive

Table 3
Differential characteristics of selected nitrogen fixing *Klebsiella* [2, 5, 8]

Characteristics	Klebsiella		
	<i>K. pneumoniae</i>	<i>K. planticola</i>	<i>K. oxytoca</i>
Gram stain	—	—	—
Motility	—	—	—
Acid and gas production in glucose	+	+	+
Gelatin hydrolysis	—	—	—
Methyl-red	—	—	—
Voges–Proskauer	+	+	+
Indole production	—	—	+
Citrate utilization	+	+	+
Urease	+	+	+
Catalase	+	+	+
Oxidase	—	—	—

(–) negative; (+) positive

Table 4
Differential characteristics of selected nitrogen fixing *Clostridium* [9]

Characteristics	Clostridium		
	<i>C. pasterianum</i>	<i>C. kluyveri</i>	<i>C. butyricum</i>
Gram stain	+	+	+
Endospore stain	+	+	+
Motility	±	+	±
Indole production	—	—	—
Starch hydrolysis	—	—	+
Nitrate reduced	—	—	—

(—) negative; (+) positive

3.3 Molecular Identification

1. Cultivate the isolates in G-YE /Jensen broth for 30 °C for 2 days.
2. Extract the genomic DNA using any standard DNA isolation kit.
3. Perform PCR amplification of 16 s rRNA gene in a total volume of 25 µl by mixing 20 ng of the template DNA with 12.5 µl of GoTaq Green (M/s Promega, USA) along with universal primers [6] as given in Subheading 2.3.
4. Keep PCR conditions as initial denaturation temperature 94 °C for 4 min followed by 30 cycles of 94 °C for 30 s, annealing temperature 52 °C for 45 s, extension temperature 72 °C for 90 s, and the final extension temperature 72 °C for 10 min.
5. Perform gel electrophoresis in 1.2% agarose gel to check the amplified product along with desired DNA markers.
6. After assuring the purity of PCR product in gel, subject the PCR product for nucleotide sequencing by DNA sequencer (Sanger's DNA sequencing) from any reliable service provider.
7. BLAST the nucleotide sequencing in NCBI database to obtain the nearest related species information.

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Isolation and Characterization of Genus *Desulfotomaculum*

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Abstract

The genus *Desulfotomaculum* is a heterogeneous group, Gram-positive, spore-forming sulfate-reducing bacteria. The first isolated genus was reported as *Clostridium nigrificans* by Werkman and Weaver, which was moderate thermophilic after being described as *Sporovibrio desulfuricans*. It forms heat-resistant endospores and reduces sulfide from sulfate by using sulfate as the terminal electron acceptor. The sulfate-reducing bacteria (SRB) can significantly impact benthic nitrogen (N) cycling by inhibiting coupled denitrification-nitrification through the production of sulfide or by increasing the availability of fixed N in the sediment via dinitrogen (N₂) fixation.

Key words Sulfate-reducing bacteria (SRB), Endospore, *Desulfotomaculum*, *Sporovibrio*, Thermophilic

1 Introduction

Genus *Desulfotomaculum* is Gram-positive, spore-forming, and anaerobic. This bacteria reduces sulfide from sulfate by using sulfate as a terminal electron acceptor [1]. The heat-resistant endospore-forming and sulfate-reducing bacteria are classified in one genus, *Desulfotomaculum* [2]. The first isolate of this genus was reported as *Clostridium nigrificans*, moderate thermophilic [3]. Later on, it is described as *Sporovibrio desulfuricans* [4]. Campbell and Postgate have reported finally as *Desulfotomaculum nigrificans* [3]. After that, some other moderately thermophilic and mesophilic *Desulfotomaculum* species were isolated from various places.

Desulfotomaculum is mostly detected from rice fields, bovine rumen, and feces, and it is also reported in subsurface environments like freshwater, marine sediments, mines, oil reservoirs, and aquifers [5–8]. They belong to the phylum *Firmicutes*, class *Clostridia*, order *Clostridiales*, and family *Peptococcaceae*. *Desulfotomaculum* cells are straight, or curve-shaped rod ranging 0.3–2.5 × 2.5–15 μm with rounded or pointed ends. Furthermore, their spore is central to terminal round or oval in shape and forming

swelling to the cell. There are 30 species and 1 subspecies reported among these, 17 are thermophilic or moderately thermophilic, 3 are halophilic, and 1 is alkaliphilic. Some species can use other sulfur-containing inorganic compounds like thiosulfate, sulfite, and elemental sulfur as terminal electron acceptors instead of sulfate [2]. Besides that, some species can also grow in the absence of sulfate by fermentation of glucose, fructose, or pyruvate [9].

2 Materials

2.1 Isolation of *Desulfotomaculum* from the Subsurface of Landfill [10]

1. Soil sample (from landfill).
2. Hollow stem auger.
3. Anaerobic jar with the anaerobic gas pack system.
4. Test tubes.
5. Petri plates.
6. Postgate's medium C (KH_2PO_4 , 0.5 g; NH_4Cl , 1.0 g; Na_2SO_4 , 4.5 g; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.06 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 2.0 g; yeast extract, 1.0 g; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.004 g; sodium citrate, 0.3 g; sodium lactate, 3.5 g; pH 7.2; distilled water, 1 l).

2.2 Isolation of *Desulfotomaculum* from Oil Reservoir [11, 12]

1. Oil sample (Oil-water emulsion).
2. Tubes.
3. Petri dishes.
4. Plasma bottle.
5. Butyl rubber stopper.
6. Anoxic medium (Na_2SO_4 , 2.0 g; KHCO_3 , 0.3 g; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 1.0 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1.0 g; 0.4% $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ solution, 1.0 ml; MOPS (morpholinepropanesulfonic acid) buffer, 3.0 g; NH_4Cl , 1.0 g; 60% sodium lactate solution, 6.0 ml; sodium acetate, 1.0 g; yeast extract 1.0 g; cysteine hydrochloride, 0.5 g; agar, 16.0 g; pH-7.4; distilled water, 1 l).

2.3 *Desulfotomaculum* Identification

2.3.1 Staining and Biochemical Methods

1. Refer to standard microbiology laboratory manual.

2.3.2 Molecular Identification

1. PCR fine chemicals as per standard protocols.
2. Primers 8F Forward (5'-AGA GTT TGA TCC TGG CTC AG-3') 1490R Reverse (5'-GGT TAC CTT GTT ACG ACT T-3').

3. Thermocycler.
4. GelDoc/transilluminator.

3 Methods

3.1 Isolation of *Desulfotomaculum* from the Subsurface of Landfill

1. Collect the wet compost soil sample (about 1.5 m) with black sediment from the landfill subsurface using the hollow stem auger technique.
2. Immediately transfer the soil sample to the laboratory in an anaerobic jar with an anaerobic gas pack system.
3. Weigh 1 g of soil sample and prepare serial dilution.
4. After serial dilution, transfer 0.1 ml of the last three dilutions on Postgate's medium C.
5. Incubate Petri plates anaerobically in nitrogen atmosphere jars for 4 days at 30 °C.
6. Purify the colonies with black precipitation from the master plate.

3.2 Isolation of *Desulfotomaculum* from the Oil Reservoir

1. Collect the oil-water emulsion sample in a sterile plasma bottle from the oil wellhead.
2. Transfer the aliquots of the aqueous phase anaerobically to SRB detection tube and then incubate it in the dark at 30 °C for 3 days.
3. Isolate the black precipitated colony and streak to agar medium.
4. Incubate the Petri dish in an anaerobic glove box with a mixture of gas containing N₂, H₂ and CO₂ (85:10:5).
5. Purify the colonies by consecutive streaking onto the same medium.

3.3 Identification of *Desulfotomaculum*

3.3.1 Staining and Biochemical Methods

1. Perform the staining technique and other biochemical tests as per standard procedures.
2. Infer the results with already available results of *Methylobacterium* (Table 1).

3.3.2 Molecular Identification [13]

1. Extract the genomic DNA from *Desulfotomaculum* as per standard protocol using the phenol–chloroform extraction method.
2. Perform PCR amplification in a total volume of 50µl by mixing 20 ng of the template DNA with 2.5 mM concentrations of each deoxynucleotide triphosphate, 1µM concentrations of

Table 1
Differential characteristics of selected *Desulfotomaculum* species

Parameters	<i>D. nigrificans</i> [14]	<i>D. defluvi</i> [10]	<i>D. thermosapovorans</i> [15]	<i>D. halophilum</i> [16]	<i>D. geothermicum</i> [17]
Gram staining	+ve	–ve	+ve	+ve	–ve
Morphology	Rod	Rod	Shape-straight/ slightly curved rod	Straight to curved rod	Straight rod
Cell size (µm)	0.3–1.5 × 3.0–15.0	2–4	1.5–2	3–6	2.3–2.5
Motility	–	–	Motile	Motile	Motile
DNA G+C content (mol%)	44.5–45.6 (LC), 46.3–46.6 (genome)	45.4 mol%	51.2 mol%	<55 mol%	50.4 mol%
Major menaquinone	MK-7	MK-7	Nr	Nr	Nr
Optimal NaCl concentration (g/l)	0	0–10%	35	40	35
Growth factor requirement	Nr/+ (yeast extract)	Malate, glycine, serine	Nr	Yeast extract	Nr
Optimum pH	6.8–7.9	7.5	7.2–7.5	6.9–8.0	7.3–7.5
Optimum temperature	55 °C	37°C	35–60°C	35°C	37–54°C
Oxidation of substrate	Incomplete	Partial	Nr	Nr	Complete
H ₂ /CO ₂	+ ^a	–	+	+	Nr
Formate	+ ^a	Nr	+	Nr	Nr
Fatty acids	–	Nr	+	Nr	Nr
Acetate	–	+	Nr	Nr	+
Ethanol	+	Nr	+	+	Nr
Lactate	+	Nr	+	+	Nr
Pyruvate	+	Nr	+	+	Nr
Fumarate	–	–	+	Nr	Nr
Succinate	–	Nr	Nr	Nr	Nr
Malate	–	Nr	Nr	+	Nr

+, positive; –, negative; Nr, not reported

^aOnly in the presence of acetate

each primer 8F (5'-AGA GTT TGA TCC TGG CTC AG-3')
 1490R (5'GGT TAC CTT GTT ACG ACT T-3') and 0.3 U of
Taq DNA polymerase in 10× *Taq* buffer.

3. Amplification reaction condition includes initial denaturation of 92 °C for 2 min followed by 30 cycles of 92 °C for 1 min, 55 °C for 1 min and 72 °C for 2 min, and then final extension at 72 °C for 5 min.
4. Purify PCR product using any PCR purification kit.
5. Identify the *Desulfotomaculum* by sequencing the 16S rDNA PCR product to ascertain the identity.

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Isolation and Identification of Associative Symbiotic N₂ Fixing Microbes: *Desulfovibrio*

Harsh Mistry, Rashmi Thakor, and Himanshu Bariya 

Abstract

Desulfovibrio species known for its sulfur-reducing characteristics plays very specific and significant role in biological fixation of nitrogen. They also help in plant growth promotion. In this chapter, easy and detailed steps have been described for the isolation and identification of *Desulfovibrio* spp. from rice soil sample. Specific biochemical characterization for identification was also performed. For species level, molecular identification using 16S rDNA sequencing with specific primers has also been described.

Key words *Desulfovibrio* spp., Isolation, 16S rDNA amplification, Sequencing, Nitrogen fixation

1 Introduction

For plant growth and development, Nitrogen is a vital limiter [1]. It is an important constituent of chlorophyll, the most impactful pigment for the process called photosynthesis, as well as amino acids and key building blocks for proteins. Nitrogen is also a key factor in other important biomolecules, such as ATP and nucleic acids. Albeit it is one of the most abundant elements mainly in the form of nitrogen gas (N₂) in the Earth's atmosphere, plants can only use reduced forms (ammonia) of this element [2].

Desulfovibrio is the genus of vibrio (curved-rod or comma) shaped, motile and non-spore forming bacteria. It is Gram-negative in nature and positive for desulfovibridin. Desulfovibridin is a dissimilatory sulfite reductase involved in energy conservation by reducing sulfite [3]. Genus *Desulfovibrio* consists of sulphate-reducing bacteria but studies suggest that among them some of these sulphate-reducing bacteria also possess ability of nitrogen fixation. *Desulfovibrio diazotrophicasp. nov.* [4], *Desulfovibrio desulfuricans* [5], *Desulfovibrio gigas*, and *Desulfovibrio vulgaris* [6] are the bacterial species from *Desulfovibrio* genus which can fix the nitrogen significantly.

2 Materials

2.1 Media for *Desulfovibrio* *desulfuricans* Isolation

Prepare agar media as described in Table 1. Autoclave media at 121 °C and 15 psi pressure for 15 min to avoid other microbial contaminations.

2.2 Isolation of *Desulfovibrio* from Soil

1. Rice paddy soil sample.
2. Gloves.
3. Sterilized sample collection containers.
4. Conical flasks (several volumes).
5. Wire loop.
6. Sterile petri dish.
7. Glass spreader.
8. 70% ethanol.
9. Shaker.
10. Lab coat.
11. Autoclave.
12. BOD incubator (28 ± 1 °C).

2.3 Molecular Identification (DNA Isolation, Amplification, and Sequencing)

1. 0.8% agarose gel for genomic DNA.
 - (a) Add 0.8 g agarose powder to 100 mL 1 × TAE buffer.
 - (b) Sprinkle agarose powder. Heat the flask until it gets digested.
 - (c) Cool down until it is not painful to touch and add 3 μL of ethidium bromide.
 - (d) Cast the gel in casting assembly and allow it to get solidify.
2. 1.2% agarose gel for PCR amplified DNA.
 - (a) Add 1.2 g agarose powder to 100 mL 1 × TAE buffer.
 - (b) Follow the steps from 2 to 4 mentioned above.
3. Centrifuge.
4. Agarose gel electrophoresis apparatus.
5. UV transilluminator.
6. PCR.
7. Primers (Forward and Reverse).
8. DNA sequencers and analyzers.
9. Micropipette and sterile tips.

Table 1
Media composition for isolation of *Desulfovibrio desulfuricans*

Sr. No.	Compound	Formula	Concentration (g/L or mL/L)
1	Dipotassium phosphate	K ₂ HPO ₄	0.5
2	Ammonium chloride	NH ₄ Cl	1.0
3	Sodium sulfate	Na ₂ SO ₄	1.0
4	Magnesium sulfate heptahydrate	MgSO ₄ ·7H ₂ O	2.0
5	Calcium chloride	CaCl ₂	0.1
6	Yeast extract	–	1.0
7	Ferrous sulfate heptahydrate	FeSO ₄ ·7H ₂ O	0.05
8	Ascorbic acid	C ₆ H ₈ O ₆	0.1
9	Sodium thioglycolate	C ₂ H ₃ NaO ₂ S	0.1
10	0.1% Resazurin solution	C ₁₂ H ₇ NO ₄	1.0 mL
11	Trace elements solution	–	1.0 mL
12	60% sodium lactate	–	12.0 mL
13	Agar	–	16.0

Make up to 1000 mL with distilled water [7]

10. GenElute™ PCR Clean-up kit.
11. BigDye® Terminator v 3.1 Cycle sequencing kit.

3 Method

3.1 Isolation of *Desulfovibrio* from Soil Sample

1. For the isolation of *Desulfovibrio*, paddy soil samples can be taken from the depth of 15–20 cm.
2. Collect paddy soil sample during rice growth stage in sterile sample collection container by using sterile spatula.
3. Sample must be brought immediately to laboratory and stored at 4 °C until further use.
4. Suspend 1 g soil sample into 9 mL of sterile distilled water contained in a conical flask to gain 10⁻¹ soil suspension.
5. Then gently shake soil suspension for 5–10 min.
6. Use sterile distilled water to obtain various dilutions of soil suspension.
7. Inoculate 100μL soil suspensions of each dilution into sterile petri plates containing agar media.
8. Spread the inoculum with the help of sterile glass spreader.
9. Incubate all the plates at 35 ± 1 °C in BOD incubator for 3 weeks.

10. Transfer individual black colonies onto fresh same agar medium plates supplemented with 0.25% of yeast extract, 0.25% of peptone, 0.25% of nutrient broth, and 0.25% of glucose or 0.25% of fructose to obtain pure culture.

3.2 Identification of *Desulfovibrio* spp.

1. Perform Gram-staining of isolates as per standard protocol and observe the slide under 100× in microscope.
2. Perform biochemical tests of isolates as per standard protocols (Table 2). Use readily available kits (HiMedia) for better experience.

3.3 Molecular Identification of *Desulfovibrio* spp.

The exact identification of *Desulfovibrio* at species level includes the use and study of the molecular methods. The molecular identification of *Desulfovibrio* is involved in the following steps:

1. Isolation of genomic DNA from the bacterial sample using standard protocols.
2. Amplification of the DNA fragment with PCR polymerase.
3. Bi-directional sequencing of the PCR product.
4. Alignment of sequenced data analysis in order to determine their neighboring genetics.

3.3.1 Amplification of the DNA Fragment

1. As defined in the PCR mixture, prepare the PCR mix.
2. Set the conditions of PCR amplification as specified in PCR amplification.
3. Check genomic and amplified product on 0.8% and 1.2% agarose gel.

PCR Mixture

Sr. No	Reactants	Quantity
1	DNA	1μL
2	Forward primer	400 ng
3	Reverse primer	400 ng
4	dNTPs (2.5 mM each)	4μL
5	10× TaqDNA polymerase assay buffer	10μL
6	TaqDNA polymerase (3 U/mL)	1μL
7	Water	μL
Total reaction volume		100μL

Table 2
Biochemical tests for *Desulfovibrio*

Organisms	Drug resistance			Growth							
	K	V	C	Catalase	Indole	Nitrate	on Bile	Urea	Motility	Desulfovibrindin	H ₂ S
<i>Desulfovibrio gigas</i>	S	R	R	—	—	—	+	—	—	+	+
<i>Desulfovibrio desulfuricans</i>	S	R	R	—	—	+	—	+	+	+	+
<i>Desulfovibrio vulgaris</i>	S	R	R	—	+	—	+	—	+	+	+

+ indicates positive, — indicates negative reaction. Resistance (R) or Susceptibility (S) to kanamycin (K), vancomycin (V), colistin (C). Data was obtained from Warren et al. (2005) [8]

PCR Amplification
Condition

Sr. No	Reactions	Temperature (°C)	Time	Cycle
1	Initial denaturation	98	5 min	1
2	Denaturation	98	15 s	25
3	Annealing	62	30 s	
4	Synthesis	72	10 s	
5	Extension	72	5 min	1

Primers

Primer				
Sr. No	name	Direction	Primer sequence (5'-3')	References
1	FoxF 530R	Forward Reverse	ATTAGATACCCTGGTAG G[T/A]ATTACCGCGG [T/G]GCTG	[7]

3.3.2 Sequencing
of the PCR Product

Purification of amplified PCR products using a GenElute™ PCR Clean-up kit (cat no. NA 1020-1kt).

Purify the Amplified PCR
Products

Cycle Sequencing

Use BigDye® Terminator v 3.1 Cycle sequencing kit for cycle sequencing. The BigDye Terminator v3.1 Cycle Sequencing Kit provides the required reagent components for the sequencing reaction in a ready reaction, premixed format.

Cycle Sequencing Reagent and Concentration

Sr. No	Reagents	Quantity
1	Terminator ready reaction mix v3.1	4μL
2	BigDye sequencing buffer	1μL
3	Template	15–300 ng
4	Primer	10 pM
5	Milli-Q water	Make up to 20μL

Cycle Sequencing Conditions

Sr. No	Reactions	Temperature (°C)	Time	Cycle
1	Incubate	96	1 min	–
2	Denaturation	96	10 s	25
3	Annealing	50	5 s	
4	Extension	60	4 min	
5	Hold	4	∞	–

Capillary Electrophoresis Parameters

Polymer	Array	Run module	Mobility file
POP-7™ polymer	50 cm	BDX_StdSeq50_ POP7xl	KB_3500_POP7xl_ BDTv3direct.mob

3.3.3 Alignment Analysis of the Sequenced Data to Identify Their Closest Neighbor Genetics

1. Submit raw data obtained from sequencing to NCBI to obtain accession number.
2. Download the FASTA format from NCBI.

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Chapter 11

Cyanobacteria

Dhruti Amin, Abhishek Sharma, and Sanket Ray

Abstract

Cyanobacteria are the most abundant autotrophic organisms found in a diverse environment. These bacteria are best known for fixing the free atmospheric nitrogen, producing organic substances, and maintaining the soil structure. Moreover, they are used as food, fertilizers, secondary metabolites, including exopolysaccharides, vitamins, toxins, enzymes, pharmaceuticals, and wastewater treatment. Though they have a broad range of applications, the most challenging part is the cultivation and handling of cyanobacteria cultures. The most important criteria to keep in mind while performing isolation and purification of cyanobacteria are specific site selection, appropriate media, incubation time, temperature, and light. This protocol chapter provides detailed information for isolation, production, and identification of cyanobacteria.

Key words Blue-green algae, Culture media, Isolation, Characterization

1 Introduction

Cyanobacteria are aquatic, [photosynthetic](#), and also known as blue-green algae. They are small, Gram-negative bacteria, generally unicellular, and often grow in large colonies. Cyanobacteria are composed of a broad range of bacteria about 2000 species in 150 genera, with a wide range of cell types, cellular structures, and physiological strategies that contribute to their ecological success in the [plankton](#), metaphyton, or [periphyton](#) [1]. It constitutes a phylogenetically coherent group of evolutionarily ancient has potential to carry out oxygen-evolving, water-oxidizing, plant-like photosynthesis, i.e. oxygenic [photosynthesis](#) with few exceptions, they produce chlorophyll *a* as primary photosynthetic pigment and phycobiliproteins as [light-harvesting pigments](#). They can grow using CO₂ as the sole source of carbon and fix via. Reductive pentose phosphate pathway [2]. Chroococcales, Pleurocapsales, Oscillatoriales, Nostocales, and Stigonematales are five major cyanobacteria groups categorized based on size, shape, and type of reproduction [3–5]. Cyanobacteria have gained much attention

due to a broad range of applications in agriculture and sustainable environment. Some of the highlights of their application are (a) a rich source of biologically active compounds with antiviral, antibacterial, and antifungal activities; (b) bioremediation; (c) biofertilizers; (d) associated with oil-degrading bacteria; (e) cyanobacterial hydrogen as an alternate source of energy; and (f) PHA-producing cyanobacteria [6]. This chapter provides an insight protocol for isolation and characterization of cyanobacteria from water and soil samples.

2 Materials

2.1 Isolation of Cyanobacteria from Water/Soil Sample

- Freshwater/marine water/soil sample.
- Sterile polythene or bottle (for sample collection).
- Selective (solid/liquid) medium, as shown in Table 1.

2.2 Identification of Cyanobacteria by Staining and Biochemical Methods

- Refer to standard microbiology laboratory manual.
- Light microscope.

2.3 Cell Counting

- Hemocytometer.
- Compound microscope.

2.4 Physiological Characterization

- For determination of nitrogenase activity, total nitrogen content, and pigment content: Chemicals as per standard methods.
- Spectrophotometer.

2.5 Molecular Identification

- PCR fine chemicals as per standard protocols.
- Bacterial universal primer 27F [12] with the cyanobacteria-specific reverse primer CYA781R [13] and the cyanobacteria-specific forward primers CYA106F [13] and CYA738F [14] along with the bacterial universal primer 1492R [12].
- Thermocycler.
- GelDoc/transilluminator.

Table 1
Commonly used culture media

Sr. No.	Type of medium	Composition (g/L)	Reference
1	BG 11 (with or without 1.5 g/L NaNO ₃)	NaNO ₃ (1.5 g), NaHCO ₃ (1.7 g), K ₂ HPO ₄ (0.04), MgSO ₄ 7H ₂ O (0.075), CaCl ₂ H ₂ O (0.036), Na ₂ CO ₃ (0.020), citric acid (0.006), ferric ammonium citrate (0.006), disodium magnesium EDTA (0.001), H ₃ BO ₃ (0.00286), Mn ₂ Cl ₂ 4H ₂ O (0.00181), ZnSO ₄ 7H ₂ O (0.00022), Na ₂ MoO ₄ 2H ₂ O (0.390), CuSO ₄ 5H ₂ O (0.079), and CoCl ₂ 6H ₂ O (0.0494), distilled water make up to 1000.0 mL, pH 7.5	Stanier et al. [7]
2	ASN III	NaCl (25.0), MgCl ₂ 5H ₂ O (2.0), KCl (0.5), NaNO ₃ (0.75), K ₂ HPO ₄ 7H ₂ O (0.02), MgSO ₄ 7H ₂ O (3.5), CaCl ₂ 2H ₂ O (0.5), citric acid (0.003), ferric ammonium citrate (0.003), EDTA (disodium salt) (0.0005), Na ₂ CO ₃ (0.02), trace metal mix A5 + Co * (1.0 mL) *Trace metal mix A5 + Co contains (g l ⁻¹) H ₃ BO ₃ (2.86), MnCl ₂ 4H ₂ O (1.81), ZnSO ₄ 7H ₂ O (0.222), Na ₂ MoO ₄ 2H ₂ O (0.390), CuSO ₄ 5H ₂ O (0.079), Co (NO ₃) ₂ 6H ₂ O (1.8 g), MgSO ₄ 7H ₂ O (0.5 g), KCl (0.06 g), CaCl ₂ H ₂ O (10.0 mg), NaNO ₃ (5.0 mg), K ₂ HPO ₄ 3H ₂ O (0.5 mg), NaSiO ₃ 9H ₂ O (15.0 mg), Tris (0.1 g), vitamin B12 (0.2 µg), vitamin mix S3* (1.0 mL), EDTA (disodium salt) (3.0 mg), FeCl ₃ (0.08 mg), ZnCl ₂ (5.0 µg), MnCl ₂ 4H ₂ O (0.12 mg), CoCl ₂ 6H ₂ O (0.3 µg), CuCl ₂ 2H ₂ O (0.12 µg), H ₃ BO ₃ (0.6 mg), distilled water (100.0 mL), pH after autoclaving: 7.6–7.8 1 mL of vitamin mix S3 contains: Thiamine HCl (0.05 mg), nicotinic acid (0.01 mg), calcium pantothenate (0.01 mg), p-aminobenzoic acid (1.0 µg), biotin (1.0 µg), inositol (0.5 mg), folic acid (0.2 µg), thymine (0.3 mg)	Rippka [8]
3	ASP2	Na ₂ EDTA (3.0 g/L) 10 mL, P1 metals 1.0 mL, iron solution 1.0 mL, KCl (60 g/L) 10 mL, CaCl ₂ 2H ₂ O (37 g/L) 10 mL, K ₂ HPO ₄ (50 g/L) 1.0 mL, NaCl 18 g, buffer solution 10 mL, MgSO ₄ 2.44 g, deionized water to 1.0 L Iron solution: FeCl ₃ ·6H ₂ O 1.95 g, 10 M HCl 5 mL, Deionized water to 500 mL, filter-sterilize Buffer solution: TAPS 100 g, TAPSO 100 g, deionized water to 1.0 L, pH 7.7 P1 metals: H ₃ BO ₃ 17.15 g, MnCl ₂ ·4H ₂ O 2.16 g, ZnSO ₄ ·7H ₂ O 0.334 g, CuSO ₄ ·5H ₂ O (0.1 g/5 mL)* 0.075 mL, CoCl ₂ ·6H ₂ O (0.1 g/5 mL)* 0.3 mL, Na ₂ MoO ₄ ·2H ₂ O (0.11 g/5 mL)* 1.0 mL, deionized water to 500 mL	Provasoli et al. [9]

(continued)

Table 1
(continued)

Sr. No.	Type of medium	Composition (g/L)	Reference
4	Bristol Medium	NaNO ₃ solution (25 g/L) 10 mL, CaCl ₂ ·2H ₂ O solution (2.5 g/L) 10 mL, MgSO ₄ ·7H ₂ O solution (7.5 g/L) 10 mL, K ₂ HPO ₄ solution (7.5 g/L) 10 mL, KH ₂ PO ₄ solution (17.5 g/L) 10 mL, NaCl solution (2.5 g/L) 10 mL, deionized water to 1 L	Bold [10]
5	Sea water enrichments (modify medium)	NaNO ₃ 1.5, K ₂ HPO ₄ 0.04, MgSO ₄ ·7H ₂ O 0.075, CaCl ₂ ·2H ₂ O 0.036, citric acid 0.006, ferric ammonium citrate 0.006, EDTA (disodium salt) 0.001, NaCO ₃ 0.02 Thiamine HCl, 1.0 µg; aged sea water, 75.0 mL; distilled water, 25.0 mL	Provasoli et al. [9] Bold and Wynne [11]

Note: For solid media: add 1.5% of agar

3 Methods

3.1 Isolation of Cyanobacteria from Water/Soil [7, 8]

1. Collect marine/freshwater/soil samples in sterile bottle/plastic polythene (*see Note 1*).
2. Primarily the sample is examined under light microscope for the presence of cyanobacteria.
3. In another portion of each sample, inoculate in 50 mL sterile liquid nutrient BG-11 medium and incubate in culture cabinet with the light intensity of 14/10 h light/dark cycle at 28 ± 1 °C for cultural enrichment (*see Note 2*).

3.1.1 Streak Method

1. Prepare agar plates containing selective medium.
2. Flame the inoculation needle and cool it by jabbing it into the edge of the agar medium.
3. With the help of wire loop, collect sample and streak on a plate by four streak method.
4. Incubate the plates at 25 ± 2 °C for 14/10 h light/dark cycle.
5. Observe blue-green colour colony.

3.1.2 Spreading Method

1. Inoculate 0.1 mL of diluted cyanobacterial sample mixture onto the surface of agar.
2. Spread it thoroughly with “L” glass rod.
3. Incubate the plates at 25 ± 2 °C for 14/10 h light/dark cycle.
4. Observe blue-green colour colony.

3.1.3 Pour Plate Method

1. Prepare a set of sterile 99.9 and 9 mL water blanks in 250 mL conical flasks and 20 mL tubes, respectively.
2. Add 100 mg cyanobacterial sample in 99.9 mL water blank and shake the contents thoroughly. Label it as 10^{-1} dilution.
3. Transfer 1 mL from 10^{-1} dilution to 9 mL water blank and mix it thoroughly. Label it as 10^{-2} dilution.
4. Transfer 0.1 mL of the diluted suspension to a tube containing molten marine agar medium cooled to 47°C , mix the contents, and pour into sterile petri dish.
5. Prepare 10^{-3} , 10^{-4} , 10^{-5} dilutions following similar procedure and transfer 0.1 mL suspension from each dilution in separate molten agar medium to prepare to pour plates from each dilution.
6. Allow the contents to solidify.
7. Incubate the plates at $25 \pm 2^{\circ}\text{C}$ for 14/10 h light/dark cycle.

3.2 Identification of Cyanobacteria

1. Refer to standard microbiology laboratory manual.
2. Record morphological taxonomic features such as trichomes, thickness, shape, size, and position akinetes/heterocyst under a light microscope and follow the taxonomic descriptions given in monographs Desikachary [15].

3.3 Cell Counting

1. Take algal culture from exponential phase and prepare homogenize.
2. Put 20 μL drop of culture on hemocytometer bed and put coverslip.
3. Observe under compound microscope at $4\times$, $10\times$, and $40\times$.
4. Calculate the no. of cells as per structure from grid of hemocytometer.

3.4 Physiological Characterization

3.4.1 Nitrogenase Activity

1. To assay potential cyanobacterial isolates which fixes atmospheric nitrogen (N_2 -ase activity).
2. To estimate nitrogenase enzyme activity by acetylene reduction assay technique (ARA) as per standard procedure [16].

3.4.2 Total Nitrogen Content

1. To determine total nitrogen content from cultures by Micro kjeldahl [17] using H_2SO_4 digestion for the cyanobacterial cells followed by steam distillation.
2. To estimate ammonia by titration with dilute HCl and to calculate the total protein content according to Bremner and Mulvaney [17].

3.4.3 *Pigments Content*

1. To estimate chlorophyll a (Chl a) in cultures by method of Meeks and Castenholz [18].
2. Also, estimate the amounts of cyanobacterial phycocyanin (C-PC) and cyanobacterial allophycocyanin (C-APC) from each sample by method of Bennett and Bogorad [19] and Singh et al. [20].

3.5 *Molecular Identification*

1. Extract the genomic DNA from *cyanobacteria* as per standard protocol using phenol–chloroform extraction method.
2. Perform PCR amplifications in a total volume of 50 µL by mixing 20 ng of the template DNA with 2.5 mM concentrations of each deoxynucleotide triphosphate, 1 µM concentrations of each primer, i.e., bacterial universal primer 27F [12] with the cyanobacteria-specific reverse primer CYA781R [13] and the cyanobacteria-specific forward primers CYA106F [13] and CYA738F [14] along with the bacterial universal primer 1492R [12].
3. Amplification reaction condition includes initial denaturation of 92 °C for 2 min followed by 30 cycles of 92 °C for 1 min, 55 °C for 1 min and 72 °C for 2 min, and then final extension at 72 °C for 5 min.
4. Perform gel electrophoresis to check the amplified product along with desired DNA markers.

4 Notes

1. Most important criteria for isolation of cyanobacteria are selecting appropriate sampling site (light intensity, temperature, pH, and salinity) and designing culture media and incubation condition.
2. The axenic cultures of cyanobacteria can be obtain using antibiotic treatment [21], repeated liquid subculture, fragmentation, and ultraviolet irradiation.

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Chapter 12

Pseudomonas

Sanket Ray and Harsh Patel

Abstract

Pseudomonas spp. are ubiquitous bacteria in agricultural soils and act as a multifunctional biocontrol possession against phytopathogens. They are best suited as biocontrol agents because of their diverse catabolic adaptability, efficient root-colonizing properties, and their capacity to produce a wide range of antifungal metabolites. Several *Pseudomonas* species have shown multiple plant growth-promoting attributes such as phosphate solubilization activity, indole acetic acid (IAA), siderophore, and HCN production. This chapter gives insight of isolation, characterization, and identification of *Pseudomonas* species as potential biocontrol agents.

Key words Isolation, Characterization, Rhizobacteria, *Pseudomonas*, Antagonism

1 Introduction

Pseudomonas are omnipresent bacteria, and are an aerobic, Gram-negative, gammaproteobacteria belonging to the family *Pseudomonadaceae* containing approx. 191 diversified species [1]. They are catalase positive and chemoorganotrophic, with a strictly respiratory metabolism. *Pseudomonas* influence plant and animal pathogens. Use of *Pseudomonas* spp. includes biocontrol, as a plant growth promoter, an efficient bioremediation strain, and also for production of array of antimicrobial compounds [2–4]. Evidence has highlighted many metabolites produced by *Pseudomonas* strains such as antibiotics, enzymes, and volatiles, and their key role in controlling an array of fungi-causing infections in plants has been used for biofertilizer production and formulation [5, 6]. In last decade, studies have been done using *P. fluorescens* to control *Pythium* damping-off of different crops like tomato, pea, cucumber, sugar beet, and wilt of tomato and pigeon pea [7–10]. Pandey and Chandel [11] have studied the efficacy of *Pseudomonas* against six fungi, viz., *Fusarium oxysporum*, *Alternaria alternata*, *Aspergillus niger*, *Pyricularia oryzae*, *Aspergillus flavus*, and *Erysiphe cruciferarum*. They recorded maximum inhibition of *Pyricularia oryzae*

fungal mycelia, which causes rice blast disease. A number of mechanisms are involved in biocontrol of phytopathogens like direct competition for root colonization and resources, synthesis of antibiotics and biosurfactants, production of mycolytic enzymes, and elicitation of induced systemic resistance (ISR) in the host plant against the pathogen [12]. Looking into the importance of *Pseudomonas* species, this chapter gives insight view on isolation and identification of *Pseudomonas* with importance of biocontrol agent.

2 Materials

2.1 Isolation of *Pseudomonas* from Soil and Water Sample

- Sterile polythene or container (for sample collection).
- Rhizosphere soil/water.
- Sterile dd H₂O.
- Selective media (Table 1).
- Shaker (30 °C, 120 RPM) and incubator (30 °C).

2.2 Staining and Biochemical Tests

Traditional methods for biochemical characterization of the genus *Pseudomonas* have been extensively described by Gordon et al. (1973) and Claus and Berkeley (1986) [13, 14].

2.3 Molecular Identification of *Pseudomonas* Spp.

2.3.1 Genomic DNA Isolation

- Lysis buffer.
- 0.25% (vol/vol) sodium dodecyl sulfate.
- 0.05 N NaOH.
- High-performance liquid chromatography-grade H₂O.

2.3.2 Primer Designing

- MegAlign software package.
- Access to GenBank database.

2.3.3 PCR Amplification

- 2 mM MgCl₂.
- 50 mM Trizma premix.
- Deoxynucleoside triphosphates.
- Primers (Table 3).
- Taq polymerase.
- Thermocycler.
- Software tool such as CLUSTAL-W and MEGA.

Table 1
Selective media and confirmative biochemical test for *Pseudomonas*

Selective enrichment broths and biochemical identification broths	Selective agars for detection and isolation	Nonselective broths	Nonselective agars for cultivation, enumeration, and isolation	Nonselective agars for differentiation	Diagnostic tests
Acetamide nutrient broth	Cetrimide nalidixic acid agar	Alternative thioglycollate medium	Milk agar	Blood agar (base)	Catalase test
Asparagine proline broth	Cetrimide agar	Brain heart broth	Nutrient agar	Calcium caseinate agar	DMACA indole disks test
Cetrimide broth	Pseudomonas agar base	Dey-Engley neutralizing broth	Skim milk agar	CLED agar	Kovac's reagent for indoles
Malachite green broth	Milk agar, modified according to Brown and Scott	Membrane filter rinse fluid (USP)	R-2A agar	King agar A and king agar B	Methyl red test
Methyl red Voges-Proskauer broth	Pseudomonas isolation agar	Peptone water	Tryptone glucose extract agar	Pseudomonas agar	Oxidase strips test
Motility nitrate medium		Thioglycollate broth	Tryptone glucose yeast extract agar	Tributyrin agar	
Nitrate broth		Vegitone infusion broth	Yeast extract agar	Blood agar (base)	

3 Methods

3.1 Isolation of *Pseudomonas* Strains by the Serial Dilution Method

1. Weigh 1 g of dry soil and add 9 mL of double-distilled water (dd H₂O) in a sterile test tube and shake well using vortex mixer.
2. Dilute this stock solution serially up to the dilution of 10⁻⁵.
3. Inoculate 0.1 mL of diluted sample on surface of selective media (Table 1).
4. Incubate at 30 °C for 2 days.
5. Preserve the purify colony by using standard preservation methods (*see* Notes 1 and 2).

3.2 *Pseudomonas* Identification by Staining and Biochemical Tests

1. Perform the staining technique and other biochemical test as per standard procedures.
2. Infer the results with already available results of *pseudomonas* spp. (Table 2).

3.3 Molecular Identification of *Pseudomonas* Spp.

3.3.1 Genomic DNA Isolation

1. Genomic DNA extraction is carried out from a single colony forming unit (CFU) culture mix with 20 μ L of lysis buffer containing 0.25% (vol/vol) sodium dodecyl sulfate and 0.05 N NaOH.
2. After heat treatment for 15 min at 95 °C followed by addition of 180 μ L of high-performance liquid chromatography-grade H₂O, store the lysis suspension at 20 °C.

Table 2
Morphological and biochemical characteristic of *Pseudomonas* species [15]

Characteristic	<i>P. fluorescens</i>	<i>P. aeruginosa</i>	<i>P. stutzeri</i>	<i>P. cepacia</i>
Colony elevation	Convex	Convex	Convex	Convex
Colony edge	Round	Round	Disc shaped with ridges	Pin point
Surface	Smooth	Smooth	Rough	Smooth
Gram stain	Gram negative	Gram negative	Gram negative	Gram negative
Microscopic observation	Rod shape cells	Rod shape cells	Rod shape cells	Rod shape cells
Spore formation	—	—	—	—
Motility	+	+	+	+
7% NaCl	+	NA	—	—
Fluorescent pigment	+	+	+	—
Catalase	+	+	+	+
Oxidase	+	+	+	+
Gas production	—	—	—	—
Starch hydrolysis	—	—	+	—
Gelatin hydrolysis	+	+	—	+
Dextrose utilization	+	+	+	—
Growth at 4 °C	+	+	+	+
Growth at 27 °C	+	+	+	+
Growth at 42 °C	—	+	+	+
Levan production	+	+	+	+
Arginine hydrolysis	+	+	—	+
Citrate utilization	+	+	+	+
Denitrification	—	+	+	+
Siderophore production	+	+	+	+

Table 3
16S rDNA-based primer sets for effective molecular identification of *Pseudomonas* spp. [16]

Primer	Sequence (5'-3')	Annealing temp (°C)	Location	Product size (bp)
PA-GS-F	GACGGGTGAGTAATGCCTA	54	95-113	618
PA-GS-R	CACTGGTGTTCCTTCCTATA		693-712	
PA-SS-F	GGGGGATCTTCGGACCTCA	58	189-206	956
PA-SS-R	TCCTTAGAGTGCCACCCG		1124-1144	

3.3.2 Primer Designing

1. Select the relevant 16S rDNA sequences available in the GenBank database and align the desired sequences in MegAlign software package (DNASTAR Inc., Madison, Wisconsin).
2. Based on the alignment output, putative genus- and species-specific primers can be design. Few well-documented 16S rDNA amplification primer sequences are tabulated in Table 3.

3.3.3 PCR Amplification

1. To amplify the target DNA by taking 25 µL reaction volumes, each containing 2 mM MgCl₂, 50 mM Trizma (pH 8.3; Sigma, St. Louis, Mo.), 250 µM (each) deoxynucleoside triphosphates (Promega, Madison, Wis.), 0.4 µM (each) primer, 1 U of Taq polymerase (Invitrogen, Carlsbad, Calif.), and 2 µL of whole-cell bacterial lysate, and make up to 25 µL by the addition of high-performance liquid chromatography-grade H₂O.
2. Amplification is carried out in a PCR thermocycler. Set the initial denaturization for 2 min at 95 °C for 25 cycles.
3. Each consisting of 20 s at 94 °C, 20 s at the appropriate annealing temperature, and 40 s at 72 °C. With a final extension of 1 min at 72 °C.
4. With this program, the total time for amplification of target DNA is approximately 45 min.
5. The purified PCR product is subjected to sequencing and aligned using CLUSTAL-W software, and phylogenetic tree is inferred by using the neighbor-joining method in MEGA software.

4 Notes

1. *Pseudomonas*, due to their metabolic flexibility and genetic diversity, are very common in different soil ecosystems.
2. For specific isolation of *Pseudomonas*, selective media is used, for example cetrimide, nalidixic acid, cephaloridine, penicillin G, pimaricin, malachite green, and other inhibitory agents.

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Isolation and Identification of Entomopathogenic *Bacillus* Species

Preeti Parmar, B. K. Rajkumar, and Naresh Butani

Abstract

Insect pests are one of the major threats to agricultural production. Among the diversified strategies adopted to control the different insect pests, agrochemicals are most preferred first line of defense due to their rapid effect and low cost but lead to environmental pollution, resistance, resurgence, and residue problems; while biological/microbial pesticides are eco-friendly. Further, entomopathogenic bacteria are widely promoted in biopesticides market due to rapid bacterial mass production, specificity towards insect pests, and safety and consistency in the environment. In recent era, the most successful bacterial entomopathogens are from the genus *Bacillus*, *Paenibacillus* and *Lysinibacillus*. Members of these genera produce parasporal crystal proteins (Cry) that damage the healthy gut wall and cause toxemia. Beside *Bacillus* species proved to be an effective bacterial group against insect pests. Isolation and identification of entomopathogenic *Bacillus* group requires deep understanding and sound knowledge of traditional and molecular approaches. Briefly, our chapter covers the critical key techniques to isolate and identify the entomopathogenic *Bacillus* group.

Key words Entomopathogen, *Bacillus*, Biopesticide, Cry protein

1 Introduction

The production and quality of the agricultural produce is severely affected by different insect pests. Agrochemicals effectively control the insect pests in majority of crops, and their continuous and injudicious use leads to short-term adverse effect of resurgence of secondary pests and imbalance of diversity and long-term adverse effect of resistance build up, residue in the biota and environmental pollution. In nature, the population of insects is maintained or kept in balance by predators and parasitoids or disease-causing microbes (entomopathogenic). Cook et al. (2002) highlighted the importance of antagonistic microbes to control the insect pests [1]. Numerous groups of entomopathogenic microbes were identified by the scientists that include bacteria, fungi, viruses, and nematodes. Among these groups, bacterial families viz. *Bacillaceae*,

Pseudomonadaceae, *Enterobacteriaceae*, *Streptococcaceae*, and *Micrococcaceae* are mostly used as biological control. *Bacillus* from the family *Bacillaceae* has the potential ability to withstand the adverse environment, replicate rapidly, and is highly pathogenic and specific to insects and fungal pathogens. For example, *Bacillus cereus*, *Bacillus popilliae*, *Bacillus sphaericus*, and *Bacillus thuringiensis* are lethal to a range of insects [2]. *B. thuringiensis* subspecies *kurstaki* [3], *B. thuringiensis* subspecies *aizawai* [4], *B. thuringiensis* subspecies *tenebrionis* [5], *B. thuringiensis* subspecies *israelensis* [6], *B. thuringiensis* subspecies *japonensis* strain Buibui [7], and *Lysinibacillus sphaericus* [8] are commercially available biocontrol microbes effective against *Lepidoptera*, *Diptera*, *Coleoptera* as well as invertebrates. Besides, *B. subtilis*, *B. megaterium*, *B. amyloliquefaciens*, *B. cereus*, and *B. pumilus* have an ability to control numerous fungal pathogens of different plant species [9, 10]. *Bacillus* is the most divergent group and the well-known biocontrol agent applied in the agriculture field. Isolation of entomopathogenic *Bacillus* species from different natural sources might provide insights to develop the new formulation of bio-insecticides. Thus, this chapter focuses on techniques to isolate and identify the *Bacillus* species used as biological control agent.

2 Materials

2.1 Pretreatment of Samples and Isolation of *Bacillus* Species

For pretreatment, sterile bags, 0.25 M sodium acetate, 0.5% Tween 80, and 0.22µm membrane filters.

For isolation, nutrient agar, selective media (J-agar, MYPGP medium, and T3 medium), and sporulation medium (MBS medium, UG medium, and soil extract agar) [11].

2.2 Traditional Approaches for Phenotypic Characteristics

Media for biochemical and serological studies.

2.3 Modern Approaches to Identify Entomopathogenic *Bacillus* Species

T3 agar medium, Coomassie brilliant blue, PCR reagents.

Table 1**Preliminary processing of the collected samples for isolating *Bacillus* group**

Sr. No.	Type of sample	Processing of the sample	Reference(s)
1	Soil	<i>Method I</i> Weigh 2–4 g of soil samples, inoculate it into sterile 10 ml distilled water in aseptic condition, and vortex it briefly <i>Method II: Acetate method</i> Take 0.5 g of soil sample and add it into 10 ml of sterile nutrient broth containing 0.25 M sodium acetate; keep the sample for 4 h at 30 °C at 180–200 rpm	Fisher and Garczynski [12] Travers et al. [13]
2	Insect	Add 0.2–0.4 g of dead insects into 1 ml of sterile distilled water containing 0.5% tween 80. Crush and homogenize the sample to proceed for further pretreatment	Fisher and Garczynski [12]
3	Water	Filter the water sample through sterile 0.22µm membrane filters to concentrate the bacteria and spores on membrane Aseptically pick the membrane and inoculate into 10 ml of sterile distilled water, vortex properly, and homogenize the sample	Fisher and Garczynski [12]

3 Methods

3.1 Pretreatment of Samples and Isolation of *Bacillus* Species

1. Sterile bags should be used to collect the soil and semisolid samples, while sterile screw-capped autoclavable plastic or glass tubes/containers should be used to collect soil, insect, and water samples. The processing of the collected samples varies depending upon the type of sample as described in Table 1.
2. Thermal shock pretreatment of the processed soil/water/insect samples at 80 °C for 10 min enriches the endospore-forming bacilli by killing the poor heat-resistant spores, especially psychrophiles and other vegetative cells [14].
3. The samples are then diluted many fold to reduce the bacterial load and to eliminate the humic material. Keep all the tubes at room temperature to settle down the solid content.
4. Transfer 1 ml of sample into 9 ml of sterile 0.85% saline tubes to make 10^{-1} dilution and spread 0.1 ml of 10^{-1} dilution sample to sterile nutrient agar media/MBS medium or other medium. Similarly, make a series of dilutions from 10^{-1} to 10^{-7} and spread onto the medium accordingly.
5. Keep all the plate at room temperature for 24–48 h to allow the growth of bacterial colony. Purify each single bacterial colony by four flame method. Examine the plates for the bacterial growth and transfer each of the colonies into the 10 ml of

sterile nutrient/MBS/selective broth and keep it for 24–48 h at 30 °C at 250 rpm on shaker to cultivate and to perform other microbiological test to identify the bacteria.

3.2 Traditional Approaches for Phenotypic Characteristics

1. 16S rDNA sequences are very similar in the group of *Bacillus subtilis* with the species *Bacillus subtilis* subsp. *subtilis*, *Bacillus amyloliquefaciens*, *Bacillus licheniformis*, *Bacillus atrophaeus*, *Bacillus mojavensis*, *Bacillus vallismortis*, *Bacillus subtilis* subsp. *spizizenii*, and *Bacillus sonorensis* [15]. Thus, combination of the traditional and molecular techniques needs to be employed for the proper identification and distinguish the *Bacillus* species as given in Fig. 1.
2. Key techniques for the agriculturally important *Bacillus* group especially useful in biocontrol of insect pests are hanging drop method to observe the motility, Gram reaction, spore staining, and other cultural and biochemical test described in Fig. 1 should be performed as per standard microbiological techniques.
3. Serotyping is the best method to differentiate the *Bacillus* species. For that, inoculate the bacterial colony into sterile nutrient medium and keep it for 5–6 h at 30 °C on shaker. Add 90 µl of bacterial culture into micro-titer plates for the determination of flagellar antigen or H antigen.
4. Prepare two dilutions of antisera (1:10 and 1:20) and mix it with 90 µl of bacterial suspension in well by which the final concentration of antisera becomes 1:100 and 1:200. For negative control, add 150 mm NaCl in deionized water instead of antisera.
5. Keep the plates at 37 °C for 75 min and observe the agglutination under compound microscope. Clear floccular sediments with clear supernatant indicate a positive reaction.

3.3 Modern Approaches to Identify Entomopathogenic *Bacillus* Species

3.3.1 Identification of Parasporal Crystals [16]

1. To identify parasporal crystals, streak *Bacillus* culture to T3 agar medium and keep it at 30 °C for 90 h.
2. Observe the colony characteristics under light microscope. After observation, prepare the bacterial smear and dip it into Coomassie brilliant blue solution for 3 min, later thoroughly wash with tap water, and examine the smear under light microscope.
3. Released crystals appear violet in color with unique shapes, spores appear white in color while vegetative cells appear as purple rods. Beside this, intracellular crystal and spores appear as white bodies within the purple bacterial cell.
4. To observe the type of crystal protein, SDS-PAGE technique is used to purify the crystal protein. Pattern of the protein is useful to differentiate the species of *Bacillus*.

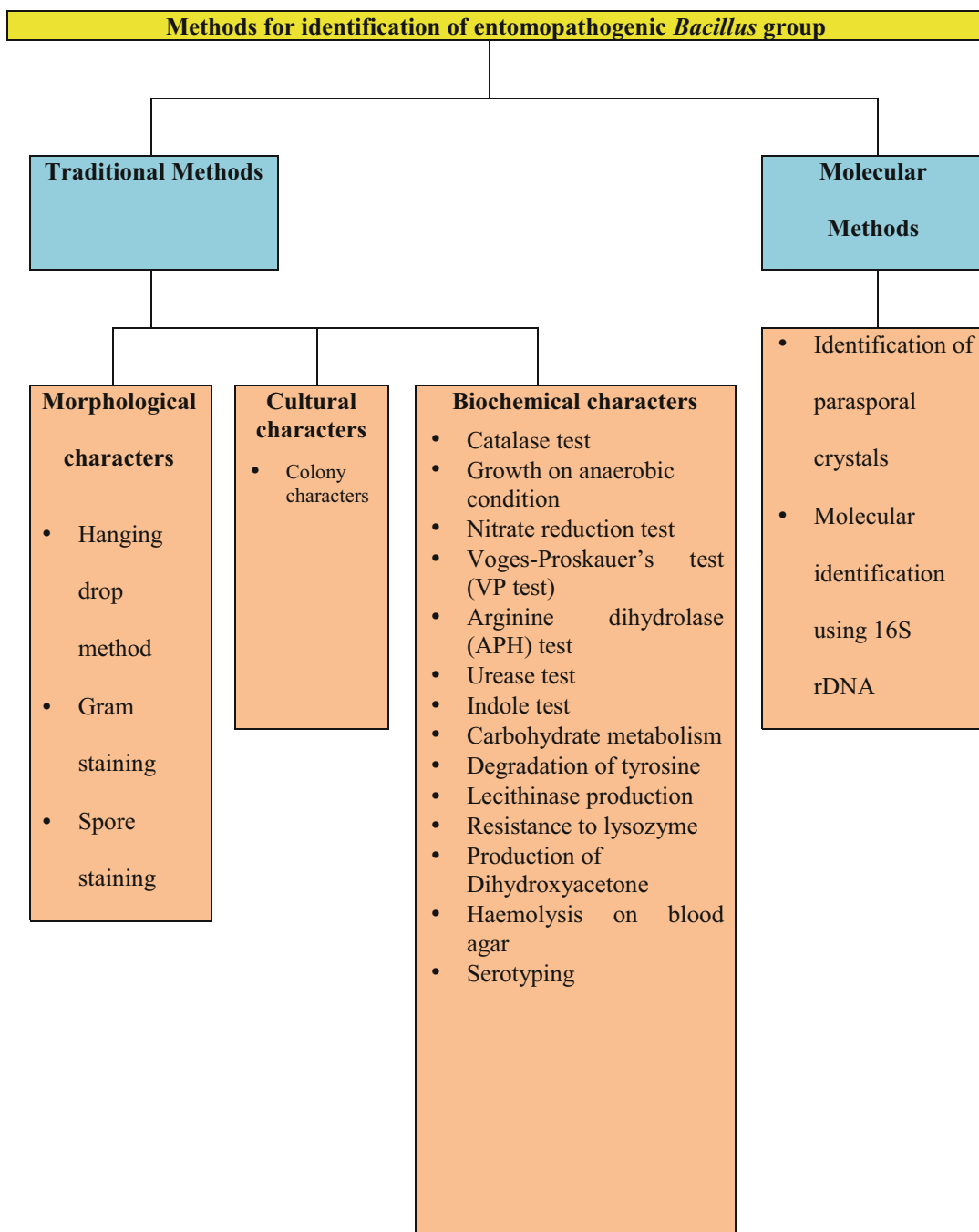


Fig. 1 Traditional and molecular methods for identification of entomopathogenic *Bacillus* group

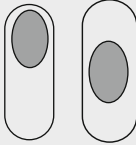
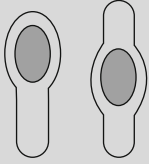
3.3.2 Molecular Identification of Bacilli Using 16S rDNA Gene Sequences

1. Molecular techniques determine the species identification of *Bacillus* genus. Bacterial DNA isolation and amplification of DNA should be performed according to standard protocols of biotechnology.

4 Notes

1. Soil sample treated by acetate method should be kept at 80 °C for 3 min [17].
2. Generally, spores of many *Bacillus* species grow rapidly on the simple nutrient medium but few vigorous species viz., *B. macerans* and *B. polymyxa*, require special medium/media after heat treatment processing [12].
3. Observation of crystals through scanning electron microscope (SEM) clearly distinguishes the type of parasporal crystals [16].
4. In classification, entomopathogenic bacteria are present in the domain *Eubacteria* that possess three divisions; Gram-negative-type cell wall (Gracilicutes), Gram-positive-type cell wall (Firmicutes) and *Eubacteria*-lacking cell wall (Tenericutes) [18].
5. It requires both traditional and molecular approaches to separate the species of the bacterial genus [18].
6. *Bacillus* group bacterial cells are Gram-positive rod shaped with the variable size from 0.4 to 1.8µm and lengths from 0.9 to 10µm. On the basis of morphology of spore shape and swelling of sporangium, Gorden et al. (1973) distinguished *Bacillus* into three distinct groups which are given in Table 2 [19].
7. Some species of *Bacillus* show swarm or rhizoid colony. Cultural colonies vary depending upon the type of medium and environmental condition [20].
8. Identification of spores is the principal key for the selection of biochemical test to differentiate the species of *Bacillus*. For example, in *B. thuringiensis*, *B. cereus*, *B. mycoides*, *B. anthracis*, *B. megaterium*, *B. licheniformis*, *B. coagulans*, *B. pumilus*, *B. subtilis*, *B. firmus*, *B. lentus*, and *B. badius* of group I can be differentiated on the basis of size and width of cell, AMC test, growth in anaerobic condition, nitrate reduction, ADH, starch utilization, gelatin, mannitol and glucose utilization, and crystal formation. Among them, *B. thuringiensis* and *B. cereus* are well-studied entomopathogens that are identified on the basis of crystal formation. Similarly, other groups of *Bacillus* identified or separated according to positive or negative tests are given in Fig. 2.
9. Group II *Bacilli* viz., *B. brevis*, *B. laterosporus*, *Paenibacillus popilliae*, *P. larvae* subsp. *Larvae*, *P. alvei*, *B. coagulans*, *B. circulans*, *P. macerans*, and *P. polymyxa*, are firstly identified based on production of gas from glucose, later differentiated and identified on the basis of growth under anaerobic condition, AMC test, L-arabinose, xylose, catalase, and indole test (Fig. 3).

Table 2**Group of *Bacillus* on the basis of spore shape and swelling of sporangium**

Sr. No.	Spore groups	Type of spores	Picture of spores	<i>Bacillus</i> group
1	Group I	Shape: Ellipsoidal or spherical endospores Position: Central or terminal Sporangia: Not swollen		<i>B. thuringiensis</i> <i>B. cereus</i> <i>B. mycoides</i> <i>B. anthracis</i> <i>B. licheniformis</i> <i>B. pumilus</i> <i>B. subtilis</i> <i>B. firmus</i> <i>B. lentus</i> <i>B. badius</i>
2	Group II	Shape: Ellipsoidal or spherical, sometimes cylindrical Position: Central or terminal Sporangia: Swollen		<i>B. brevis</i> <i>B. laterosporus</i> <i>Paenibacillus popilliae</i> <i>Paenibacillus larvae</i> subsp. <i>Larvae</i> <i>Paenibacillus alvei</i> <i>B. coagulans</i> <i>B. circulans</i> <i>Paenibacillus macerans</i> <i>Paenibacillus polymyxa</i>
3	Group III	Shape: Round or nearly round Position: Terminal or subterminal Sporangia: Swollen		<i>Sporosarcina pasteurii</i> <i>Lysinibacillus sphaericus</i>

10. Further, group III *Bacilli*, *Sporosarcina pasteurii* and *Lysinibacillus sphaericus*, are identified based on growth under anaerobic condition and glucose and nitrate reduction test (Fig. 4). Biochemical characteristics of *Bacillus* species are given in Tables 3, 4, and 5 while Figs. 2, 3, and 4 show the key biochemical test results to distinguish *Bacillus* species of each group.
11. Flagellar antigens are also helpful to differentiate the crystal protein producing *Bacillus* species. For example, besides *B. thuringiensis*, some strains of the *Lysinibacillus sphaericus* previously known as *B. sphaericus* produce a parasporal binary protein (Bin) and/or mosquitocidal toxin (Mtx) that is toxic to mosquito larvae (*Anopheles* and *Culex* species) [21]. Here, *L. sphaericus* is strictly aerobic bacillus that produces both spore and parasporal bodies inside the exosporium while

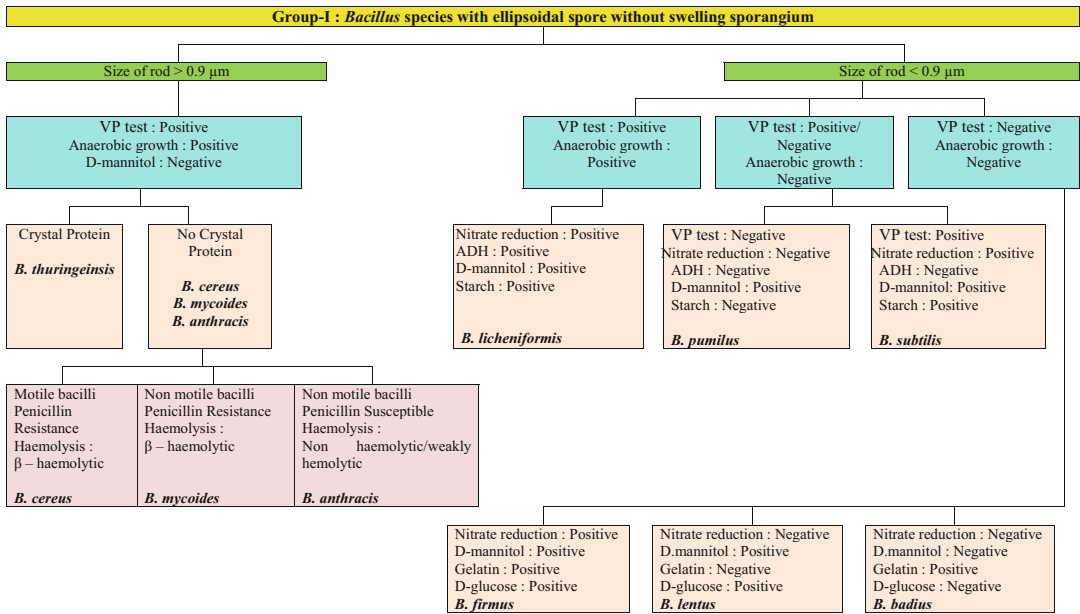


Fig. 2 Tests to differentiate group I *Bacillus* species

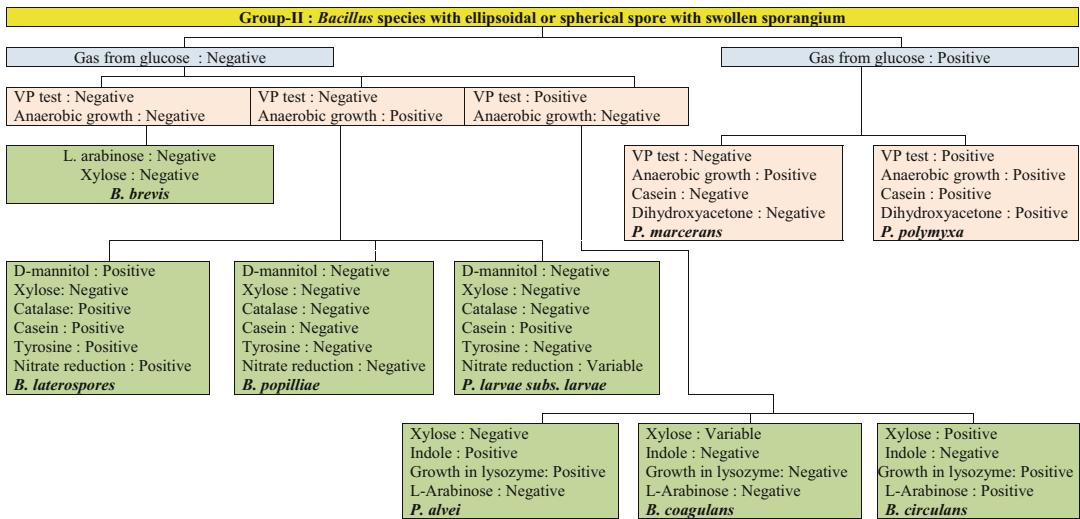


Fig. 3 Differentiation of group II *Bacillus* species

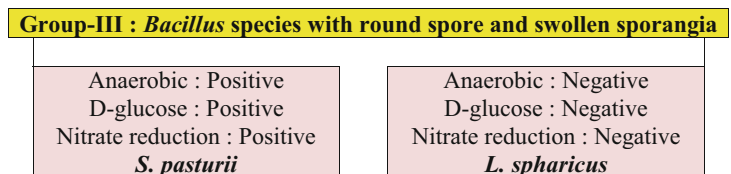


Fig. 4 Tests to differentiate group III *Bacillus* species

Table 3
Biochemical test of Group I *Bacillus* species

Test	<i>B. thuringiensis</i>	<i>B. cereus</i>	<i>B. mycoides</i>	<i>B. anthracis</i>	<i>B. licheniformis</i>	<i>B. pumilus</i>	<i>B. subtilis</i>	<i>B. firmus</i>	<i>B. lentus</i>	<i>B. badius</i>
Lecithinase	Positive	Positive	Positive	Positive	Negative	Negative	Negative	Negative	Negative	Negative
Acid from glucose	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Negative
L-arabinose	Negative	Negative	Negative	Negative	Positive	Positive	Positive	Negative	Positive	Negative
D-xylose	Negative	Negative	Negative	Negative	Positive	Positive	Positive	Negative	Positive	Negative
Hydrolysis of starch	Positive	Positive	Positive	Positive	Positive	Negative	Positive	Positive	Positive	Negative
Casein	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Variable	Positive
Nitrate reduction	Positive	Positive	Positive	Positive	Positive	Negative	Positive	Variable	Variable	Negative
Degradation of tyrosine	Not done	Positive	Not done	Variable	Negative	Negative	Negative	Variable	Negative	Positive
Citrate utilization	Positive	Positive	Variable	Variable	Positive	Positive	Positive	Negative	Negative	Negative

Table 4
Biochemical test of Group II *Bacillus* species

Group II: Ellipsoidal or spherical spore with swollen sporangium <i>Bacillus</i> group										
Test	<i>P. larvae</i> subsp.									
	<i>B. brevis</i>	<i>B. laterosporus</i>	<i>P. popilliae</i>	Larvae	<i>P. alvei</i>	<i>B. coagulans</i>	<i>B. circulans</i>	<i>P. macerans</i>	<i>P. polymyxa</i>	
Catalase	Positive	Positive	Negative	Negative	Positive	Positive	Positive	Positive	Positive	Positive
Anaerobic growth	Negative	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive
VP test	Negative	Negative	Negative	Negative	Positive	Positive	Negative	Negative	Positive	Positive
Growth in lysozyme	Variable	Positive	Positive	Positive	Positive	Negative	Positive	Negative	Variable	Variable
Glucose	Variable	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive
Arabinose	Negative	Negative	Negative	Negative	Negative	Negative	Positive	Positive	Positive	Positive
Xylose	Negative	Negative	Negative	Negative	Negative	Variable	Positive	Positive	Positive	Positive
Mannitol	Variable	Positive	Negative	Negative	Negative	Variable	Positive	Positive	Positive	Positive
Gas from glucose	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Positive	Positive	Positive
Starch	Negative	Negative	Negative	Negative	Positive	Positive	Positive	Positive	Positive	Positive
Casein	Positive	Positive	Negative	Positive	Positive	Variable	Negative	Negative	Positive	Positive
Indole	Negative	Variable	Negative	Negative	Positive	Negative	Negative	Negative	Negative	Negative
Dihydroxyacetone	Negative	Negative	Negative	Negative	Positive	Variable	Negative	Negative	Positive	Positive
Tyrosine	Positive	Positive	Negative	Negative	Variable	Negative	Negative	Negative	Negative	Negative
Nitrate reduction	Variable	Positive	Negative	Variable	Negative	Variable	Negative	Positive	Positive	Positive
Citrate	Variable	Negative	Negative	Negative	Negative	Variable	Negative	Variable	Negative	Negative

Table 5
Biochemical test of Group III *Bacillus* species

Group III: Round spore and swollen sporangia <i>Bacillus</i> species		
Tests	<i>Sporosarina pasteurii</i>	<i>Lysinibacillus sphaericus</i>
Anaerobic growth	Positive	Negative
Glucose	Negative/positive	Negative
Urea	Positive	Positive
Arabinose	Negative	Negative
Xylose	Negative	Negative
Mannitol	Negative	Negative
Starch	Negative	Negative
Casein	Negative	Variable
Nitrate reduction	Positive	Negative

B. thuringiensis is aerobic or facultative aerobic that produces inclusions (crystals) during sporulation.

12. To identify the Bt protein producing strains, first observe the bacterial colony. Crystal and spore producing colonies are generally white, depressed with rough edge while nonsporulating colonies are opaque, raised and smooth margin. Basically, bipyramidal, cuboidal, and spherical-shaped crystal appears with basic staining or with scanning electron microscope [16].
13. SDS-PAGE analysis of Bt or crystal protein is useful to predict the presence as well as diversity in cry genes. Lepidopteran-active proteins are of 130–138 kDa encoded by Cry1 genes while Cry2, Cry3, Cry4, and Cry10 or Cry11 are of 65, 70, 135, and 80 kDa, respectively [22]. Crystals of the *L. sphaericus* strains comprise two equimolar insecticidal proteins Bin A and Bin B with 41.9 and 51.3 kDa size, respectively, that share no homology with cry proteins of *B. thuringiensis* [23].
14. The 16S rDNA gene is approximately 1.5 kb and is composed of regions with different levels of variability thus providing valuable information for differentiation of taxa and also determines phylogenetic relationships of novel bacterial isolates/species. Identity scores of 97% and 99% were considered for the identification at genus and species level, respectively. Further, strains that show less than 97% 16S rDNA sequence are considered to be new and/or different species [24].

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Chapter 14

Methylobacterium

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Abstract

Pink-pigmented methylotrophic bacteria are methanol utilizers universally found in multifarious environments. Methylotrophs are ample colonizer of the phyllosphere because the leaves emit methanol during cell division as a waste product. Methylotrophs are reported for the mitigation of agricultural stress and to overcome the greenhouse gas. Leaf-impression method is commonly used for the isolation of methylotrophic bacteria from the phyllosphere. Methylotrophs are identified based on staining methods, biochemical test, and molecular identification of *mxaf* gene of methanol dehydrogenase.

Key words Methanotrophs, Methanol, AMS medium, Methanol dehydrogenase, Pink pigmented

1 Introduction

Methanotrophs or methanotrophic bacteria are a subset of a physiological group of bacteria called as methylotrophs. Methylotrophs are ubiquitous in nature and found in various environment including water, air, soil, and plant. This bacterium has unique ability to utilize methanol as a sole carbon and energy source. Methylotrophs are aerobic, heterotrophic microflora frequently found on surface of the leaves of different plants species. These groups of bacteria are important to overcome the global warming through taking up greenhouse gas methane and also actively playing a role in carbon cycling in soil [1]. *Methylobacterium* is pink-pigmented methylotrophic bacteria (PPFMs), and it is abundant colonizer of the phyllosphere due to availability of methanol on the leaf surface. Methanol is a waste product of the pectin metabolism emitted during cell division.

The *Methylobacterium* was first isolated by Basile et al. [2] as contaminants from the tissue culture of liverwort, and *Scapania nemorosa*, later identified as the genus *Methylobacterium*. Methylotrophs are mainly two types: obligate methylotrophs; can only able to grow on C1 substrate, and facultative methylotrophs; can grow

on C1 substrate as well as multicarbon compounds such as diethyl ether, dimethylamine, trimethylamine, tetramethylamine, trimethylsulfonium, trimethylamine N-oxide, and some other organic compounds like acetate, lactate, pyruvate, and carboxylic acid. *Methylobacterium* species have been reported as PGPB due to its ability to synthesize phytohormones [3] and antagonistic activity against pathogens [4].

2 Materials

2.1 Isolation of *Methylobacterium* from Phyllosphere

- Healthy fresh leaves.
- Sterile polythene (for sample collection).
- Methanol.
- Distilled water (washing purpose).
- AMS medium (K_2HPO_4 , 0.7 g; NH_4Cl , 0.54 g; $ZnSO_4 \cdot 7H_2O$, 0.1 mg; $MnCl_2 \cdot 4H_2O$, 0.3 mg; H_3BO_3 , 0.3 mg; $CoCl_2 \cdot 6H_2O$, 0.2 mg; $CuCl_2 \cdot 2H_2O$, 0.01 mg; $NiCl_2 \cdot 6H_2O$, 0.02 mg; $Na_2MoO_4 \cdot 2H_2O$, 0.06 mg; agar, 15 g; distilled water, 1.0 L; pH 6.8).
- Spatula.
- Glycerol.

2.2 Isolation of *Methylobacterium* from Soil Sample

- Soil sample.
- Shaker (at 28 °C) and incubator (30 °C).
- Antifungal (cycloheximide or amphotericin B *see* Note 1).
- Other materials as mentioned above.

2.3 Isolation of Endophytic *Methylobacterium*

- Stem, root, and leaf.
- 250 ml Erlenmeyer flask.
- 70% ethanol.
- Methanol.
- Sodium hypochlorite.
- Shaker.
- Glycerol.

2.4 Methylotrophic Bacteria Identification

2.4.1 Staining and Biochemical Methods

- Refer standard microbiology laboratory manual.

2.4.2 Electron Microscopic Analysis

- 0.05 M sodium cacodylate buffer.
- 1% (v/v) glutaraldehyde.
- 1% (w/v) OsO₄.
- 0.05 M cacodylate buffer.
- Alcohol.
- Spurr epoxy resin.
- 1% uranyl acetate.
- 2% lead citrate.
- 0.3% (w/v) phosphotungstic.
- Electron microscope.

2.4.3 Molecular Identification (see **Note 2**)

- PCR fine chemicals as per standard protocols.
- Primers *mxn* fl003 (5'-GCG GCA CCA ACT GGG GCT GGT-3') and *max* r1561 (5'-GGG CAG CAT GAA GGG CTC CC-3') [5].
- Thermocycler.
- GelDoc/transilluminator.

3 Methods

3.1 Isolation of Methylobacterium from Phyllosphere (Leaf-Imprinting Technique)

1. Collect healthy leaves in a sterile plastic bag and transfer to the lab.
2. Wash the collected leaf sample with distilled water to remove dirt and soil from the leaf surface.
3. Gently press the leaf sample onto the AMS agar medium plates supplemented with 0.5% methanol.
4. After the leaf-impression, remove the leaf carefully with the help of spatula from the surface of the medium and discard it.
5. Incubate the plates at 30 °C for up to 2 weeks as they are relatively slow growers (till the distinct pink color colony appears).
6. Purify the colonies from the master plate and store at –80 °C as a glycerol stock.

3.2 Isolation of *Methylobacterium* from Soil Sample

1. Collect the soil sample in sterile ziplock bag and transfer to the lab.
2. Weigh 10 g of soil sample and add into 90 ml of distilled water and keep it on rotary shaker at 150 rpm for 30 min.
3. From the soil suspensions, prepare tenfold serial dilution and plate 0.1 ml of last three dilutions on AMS agar medium amended with 0.5% (V/V) methanol.
4. Incubate the plates at 30 °C for up to 2 weeks or till the distinct pink color colony appears.
5. Purify the pink-pigmented colony from the master plate and store at –80 °C as a glycerol stock for the further use.

3.3 Isolation of Endophytic *Methylobacterium*

1. Collect root or stem (fresh and healthy) and surface-sterilize with 70% ethanol for 1 min, followed by in sodium hypochlorite for 5 min, and then rinse twice in distilled water.
2. After the surface sterilization, macerate 10 g of the plant materials in sterile distilled water.
3. From the maceration, collect 10 ml of sample and mix it with flask containing 90 ml distilled water.
4. Keep the flask on the rotary shaker at 150 rpm at 22 °C for 1 h.
5. Plate 0.1 ml of suspension on to the AMS agar plate medium amended with 5% (v/v) methanol.
6. Incubate the plates at 30 °C for up to 2 weeks. After incubation, select the pink-pigmented colonies from master plates and purify as per standard procedure.
7. Store the purified strain as a glycerol stock at –80 °C for the further study.

3.4 Methylotrophic Bacteria Identification

1. Perform the staining technique and other biochemical test as per standard procedures.
2. Infer the results with already available results of *Methylobacterium* (Table 1).

3.5 Electron Microscopic Analysis

1. To obtain thin sections of culture cells, fix the bacterial cells for 1 h at 4 °C in 0.05 M cacodylate buffer (pH 7.2, 1% (v/v) glutaraldehyde).
2. After 1 h, wash three times in the same buffer and refix in 1% (w/v) OsO₄ in 0.05 M cacodylate buffer for 4 h at 4 °C.
3. After dehydration in a series of alcohols, inoculate cells in spurr epoxy resin and section with an LKB 2128 Ultratome.
4. Stain the thin section with 1% uranyl acetate and then with 2% lead citrate for 10 min and mount on copper grids.
5. Stain the cell with 0.3% (w/v) phosphotungstic acid (pH 7.2).

Table 1
Differential characteristics of selected *Methylobacterium* species

Characteristics	<i>M. populi</i> [6]	<i>M. fujiisawaense</i> [7]	<i>M. radiotolerans</i> [8]	<i>M. zatmanii</i> [9]	<i>M. mesophilicum</i> [9]	<i>M. aminovorans</i> [6]	<i>M. oryzae</i> [10]	<i>M. organophilum</i> [6]	<i>M. extorquens</i> [10]	<i>M. rhodesianum</i> [11]
D-glucose	–	+	+	–	+	–	–	–	–	–
D-Fucose	–	+	+	–	+	ND	+	–	–	–
D-xylose	–	+	+	–	–	–	+	–	–	–
L-arabinose	–	+	+	–	+	ND	–	–	–	–
Fructose	+	–	–	+	–	+	–	+	–	+
Galactose	+	+	+	+	+	+	+	–	+	+
L-aspartate/L-glutamate	–	+	+	–	+	+	+	+	V	V
Citrate	–	+	+	–	+	–	+	–	–	–
Sebacate	–	+	+	–	V	ND	+	–	–	–
Acetate	+	+	+	+	–	+	–	+	+	+
Betaine	+	–	+	–	–	+	–	–	+	+
Tartrate	+	V	–	V	–	ND	–	–	+	–
Ethanol	+	+	V	+	+	ND	+	+	+	+
Methane	+	–	–	–	–	ND	–	V	+	+
Methylamine	+	–	–	–	+	+	–	+	+	+
Dimethylamine	–	–	–	+	–	+	+	–	–	+
Trimethylamine	–	–	–	–	–	+	+	+	–	–
Cyanate	–	ND	ND	ND	ND	ND	–	ND	ND	ND
Thiocyanate	–	ND	ND	ND	ND	ND	–	ND	ND	ND
Nutrient agar	+	+	+	+	–	ND	+	+	V	+
Optimal temperature	20–30 °C	15–37 °C	15–37 °C	20–37 °C	30–42 °C	28 °C	20–30 °C	28 °C	30 °C	15–37 °C
Gram reaction	–	–	–	–	–	–	–	–	–	–
Motility	+	+	+	+	+	+	+	+	+	+

(continued)

Table 1
(continued)

Characteristics	<i>M. populi</i> [6]	<i>M. fujiisawaense</i> [7]	<i>M. radiotolerans</i> [8]	<i>M. zatmanii</i> [9]	<i>M. mesophilicum</i> [9]	<i>M. aminovorans</i> [6]	<i>M. oryzae</i> [10]	<i>M. organophilum</i> [6]	<i>M. extorquens</i> [10]	<i>M. rhodesianum</i> [11]
Spore forming	–	–	–	–	–	–	–	–	–	–
Oxidase	+	–	+	+	+	+	+	+	+	+
Oxidation of methanol	+	+	+	+	+	+	+	+	+	+
Catalase	+	+	+	+	+	+	+	+	+	+
Urease reduction	+	+	+	+	+	+	+	+	+	+
Indole and methyl red	–	–	–	–	–	–	–	–	–	–
Voges-Proskauer test	–	–	–	–	–	–	–	–	–	–

V variable result, – negative, + positive, ND not determined

6. Take the micrograph in a transmission electroscope at an operating voltage of 60 kV.

3.6 Molecular Identification

1. Extract the genomic DNA from *Methylobacterium* as per standard protocol using phenol–chloroform extraction method.
2. Perform PCR amplifications in a total volume of 50 µl by mixing 20 ng of the template DNA with 2.5 mM concentrations of each deoxynucleotide triphosphate, 1 µM concentrations of each primer *mxα* f1003 (5'-GCG GCA CCA ACT GGG GCT GGT-3') and max r1561 (5'-GGG CAG CAT GAA GGG CTC CC-3') described by McDonald and Murrell [5] and 0.3 U of *Taq* DNA polymerase in 10× *Taq* buffer.
3. Amplification reaction condition includes initial denaturation of 92 °C for 2 min followed by 30 cycles of 92 °C for 1 min, 55 °C for 1 min and 72 °C for 2 min, and then final extension at 72 °C for 5 min.
4. Perform gel electrophoresis to check the amplified product along with desired DNA markers.

4 Notes

1. Add filter sterilized cycloheximide or amphotericin B in the AMS media to avoid any fungal growth.
2. Use freshly prepared solution.

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Isolation and Identification of *Beijerinckia*

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Abstract

Genus *Beijerinckia* found widely in the acidic tropical soil and phyllosphere of tropical plants and aerobically fix nitrogen. *Beijerinckia* spp. are reported for the establishment of microbes and as a plant growth promoter. Nitrogen-free, enriched media is commonly used for the isolation of *Beijerinckia* from soil sample and leaf sample. They formed a smooth, slimy colony on the nitrogen-free agar medium; they formed intracellular polyhydroxybutyrate (PBH) granules at both poles.

Key words *Beijerinckia*, Ecology, Nitrogen-free enrichment media, Preservation, Identification

1 Introduction

Genus *Beijerinckia* were initially isolated from acidic soils (pH 4.5, 4.9, and 5.2) by Altson [1] and Starkey and De [2]. Later on, the *Beijerinckia* genus was found to be widely in the acidic tropical soils of Africa, South America, and South Asia [3, 4]. *Beijerinckia* strain was also reported in neutral, alkaline, acidic volcanic ash, water-logged soils, and nontropical soils [4–11].

Beijerinckia cells are Gram-negative straight or slightly curved rod around $0.5\text{--}2.0 \times 1.6\text{--}4.5\mu\text{m}$ or sometimes $3.0 \times 5.6\text{--}6.0\mu\text{m}$ in size. They are aerobic, motile by peritrichous flagella, fix N_2 under aerobic or microaerobic conditions, optimum growth temperature at $20\text{--}30^\circ\text{C}$ and no growth at 37°C . They form intracellular granules of PBH at each pole; on agar media (N_2 fixing condition) produce smooth, slimy colonies. They are catalase-positive, oxidize to CO_2 , and utilize glucose, fructose, and sucrose. *Beijerinckia* species are commonly restricted to acidic soil of tropical regions, they are comparatively slow-growing bacteria, and their population was marked in the rhizoplane region than in the rhizosphere. Dobereiner [12] demonstrated that *Beijerinckia* helps establish bacteria and promote plant growth and increase yield. Ruinen [13, 14] also observed that *Beijerinckia* species abundantly

colonize the phyllosphere of tropical plants. For the isolation of genus *Beijerinckia*, acidic nitrogen-free enrichment selective medium [10, 15, 16] can be used; low pH of this medium favours the growth of *Beijerinckia* species and inhibits the growth of other organisms, especially *Azotobacter* species.

2 Materials

2.1 Isolation of *Beijerinckia* Species from the Soil

- *Enrichment media*
Glucose 20.0 g; KH_2PO_4 1.0 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.5 g; pH 5.0; distilled water 1 L.
- *Nitrogen-free, glucose mineral agar medium*
Glucose 20.0 g; KH_2PO_4 0.2 g; K_2HPO_4 0.8 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.5 g; $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ 0.0025 g or 0.05 g; $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ 0.005 g; CaCl_2 0.05 g; pH 6.9; distilled water 1 L.
- Rhizospheric soil.
- Petri plates.
- Incubator (20–30 °C).

2.2 Isolation of Phyllospheric *Beijerinckia* Species

- Same as mentioned above.

2.3 *Beijerinckia* Identification

- Refer to standard microbiology laboratory manual.

2.3.1 Staining and Biochemical Test

2.3.2 Molecular Identification [17]

- TS buffer.
- TO buffer.
- Disodium EDTA.
- 15% SDS.
- 5 M NaCl.
- CsCl–ethidium bromide.
- PCR fine chemicals as per standard protocols.
- Primers 27F and 1492R.
- Thermocycler.
- GelDoc/transilluminator.

3 Methods

3.1 Isolation of *Beijerinckia* from the Rhizospheric Soil Sample (Enrichment Method [10, 15, 16])

1. Prepare thin layer (2–3 mm) medium into Petri plate. (Thin layer will allow reasonable access of O₂ and inhibit the development of anaerobic and butyric acid bacteria (nitrogen-fixing *Clostridium pasteurianum*) and facultative dinitrogen-fixing anaerobes).
2. Inoculate 0.5 g of soil (*see Note 1*) as inoculums, then incubate at 30 °C for more than a week. (In the development stage, enrichment culture changes in a viscous growth).
3. After incubation, examine enrichment culture microscopically for the presence of the characteristic *Beijerinckia* cells (blunt rods with two lipoid bodies on each end of the cell).
4. After confirmation of *Beijerinckia* cells based on microscopic examination, plate the culture on nitrogen-free mineral agar medium.

3.2 Isolation of *Phyllospheric Beijerinckia* Species

1. Collect leaf sample and submerge in a shallow layer of liquid enrichment medium in Petri plates.
2. Incubate the plate at 30 °C for more than 2 weeks.
3. After incubation, select the colony for the purification based on colony characteristics. (Colony morphology: highly raised, tough slime, and glistening colonies of very elastic).
4. Use nitrogen-free mineral agar medium (as described above) for the purification of *Beijerinckia*. (Media should be at neutral or alkaline pH with CaCO₃ (1 or 2% w/v) over CaCl₂ (*see Note 2*)). Note: On alkaline media, slime is less tenacious and it can dissolve in sterile distilled water and in medium.
5. Check isolated culture on the peptone media in contrast to *Azotobacter* and *Azomonas* species. (*Beijerinckia* will not grow on this media, and contamination will overgrow) (*see Note 3*).

3.3 *Beijerinckia* Bacteria Identification

3.3.1 Staining and Biochemical Test

- Perform staining and biochemical test as per standard protocol (Table 1).

3.3.2 Molecular Identification [17]

- Grow cells overnight in nitrogen-free media and harvest pellets using the centrifugation method.
- Suspend pellets into TS buffer (9% sucrose, 0.05 M Tris, pH 8; 10 mL of buffer for each centrifuge tube with about 1.5×10^{10} cells).
- Add disodium EDTA and lysozyme to it, then briefly mix on a vortex; incubate at 37 °C for 5 min.

Table 1
Differential characteristics of the species of *Beijerinckia*

Characteristics	<i>B. derxii</i>	<i>B. indica</i>	<i>B. doebereineriae</i>	<i>B. fluminensis</i>	<i>B. mobilis</i>
Cell size (µm)	1.5–2.0 × 3.5–4.5	0.5–1.2 × 1.6–3.0	1.0 × 3.25	1.0–1.5 × 3.0–3.5	0.6–1.0 × 1.6–3.0
Colony colour after ageing	Beige	Pink	Cream	Pink	Amber brown
Motility	–	–	–	–	+
pH range for growth	4.0–9.0 (6.0–7.0)	3.0–10.0 (4.0–10.0)	3.0–10.0 (6.5)	3.5–9.2 (ND)	3.0–10.0 (4.0–5.0)
Resistant to 1% peptone	–	d	–	–	D
Starch hydrolyzed	D	–	–	–	–
Growth on asparagine as C and N source	–	–	–	–	–
Urea hydrolyzed	+	+	–	–	+
H ₂ S production from cysteine	–	–	+	+	–
DNA G + C content (mol %)	57.5–60.7	54.7–58.8	57.1	54.4–58.0	57.3
<i>Carbon compounds utilization</i>					
Arabinose	–	+	+	D	+
Galactose	+	+	+	+	–
Fructose	+	+	+	+	–
Melibiose	D	d	–	+	–
Maltose	+	–	+	D	–
Mannose	–	+	+	D	–
Sorbose	D	+	–	+	+
Raffinose	+	+	–	D	d
Xylose	–	–	+	+	–
Fucose	–	–	+	–	–
Methanol	–	–	–	–	+

Glycerol	—	+	—	+	+
Sorbitol	+	+	—	+	—
Mannitol	+	+	—	d	d
Gluconate	+	+	—	+	+

d different among strains

- Add 15% sodium dodecyl sulfate and slowly invert tubes to mix it several times, then set at 37 °C for 5 min.
- Add 5 M NaCl and gently mix it. Place the tubes in the icebox for 10 min (can be stored at 4 °C overnight).
- Harvest the DNA through the centrifugation method and add CsCl–ethidium bromide, and again centrifuge it.
- Store the extracted DNA in the TO buffer (10 mM tris; 1 mM disodium EDTA, pH 8) at –80 °C.
- Amplify the 16S rDNA using primers 27F and 1492R [18] following standard PCR conditions.
- Sequence PCR product using dideoxy terminator cycle sequencing kit.
- BLAST analyze the obtained sequence in NCBI GenBank or Ezbiocloud software and infer the results of *Beijerinckia*.

4 Notes

1. Isolation of *Beijerinckia* from water sample is also possible, but inoculum size should be more than 10 mL. For this, use double strength liquid enrichment media (pH 5.0 or lower).
2. For the cultivation of *Beijerinckia*, CaCl can be omitted to obtain a calcium-free medium and it can be replaced over CaCO₃. Sometime calcium carbonate extends the log phase of *Beijerinckia* growth.
3. For long-term storage, lyophilize *Beijerinckia* strain in skim milk or dextran-sodium glutamate solution on filter paper, then store at room temperature in dark condition.

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Chapter 16

Isolation of *Streptomyces* from Soil Sample

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Abstract

Streptomyces is an aerobic, Gram-positive, and filamentous bacteria. The filaments yield economical use of nutrients within the rhizosphere, and they allow *Streptomyces* to colonize the substrates higher than living microorganisms. After the serial dilution of soil sample, pour-plate technique is performed using starch casein agar (SCA) medium, and inoculated plates were at room temperature for 7 days. The morphological identification of *Streptomyces* is performed by coverslip technique.

Key words *Streptomyces*, Soil sample, Starch casein agar (SCA) medium, Coverslip technique

1 Introduction

Streptomyces is a Gram-positive bacterium that can grow in various environments. It is the largest group among the actinobacteria. Actinobacteria are the widely distributed group of microorganisms in various ecological habitats such as soil, freshwater, backwater, lake, compost, sewage, and marine environment. Various physical, chemical, and geographical factors determine the diversity of actinobacteria in different environments. Their high G + C content (>55%), formation of asexual spores and branching filaments, differentiates actinobacteria from other bacterial community. They produce a number of important secondary metabolites including antibiotics (antibacterial, antifungal, and antiviral), herbicidal, anti-tumor agents, immunosuppressive agents, and enzymes. It has been stated that more than two-thirds of total naturally occurring antibiotics have been produced by actinobacteria [1], which lead to the development of newer variety of drugs [2]. Recent studies by the researchers clearly reveal that there is an increased resolution of known compounds because of the decrease in the production of novel secondary metabolites by the terrestrial actinobacteria, which led to the isolation of actinobacteria from underexploited or uncharted habitats.

The production of most antibiotics is species specific, and these secondary metabolites are important for *Streptomyces* sp. in order to compete with other microorganisms that come in contact, even within the same genus. Another necessary method involving the assembly of antibiotics is that the mutuality between *Streptomyces* and plants, because the antibiotic protects the plant against pathogens, and plant exudates permit the event of *Streptomyces* [3, 4]. In this chapter, we describe the experimental protocol for isolation and morphological identification of *Streptomyces* from soil samples.

2 Materials Required

1. Soil sample.
2. Test tubes.
3. Petri plates.
4. 1 ml micropipette and tips.
5. Distilled water.
6. *Starch casein agar* (SCA) medium composition for 1 l: Soluble starch, 10 g; casein, 0.3 g; KNO₃, 2 g; MgSO₄·7H₂O, 0.05 g; K₂HPO₄, 2 g; NaCl, 2 g; CaCO₃, 0.02 g; FeSO₄·7H₂O, 0.01 g; and agar, 18 g.

3 Methods

1. Take eight test tubes containing 9 ml of distilled water.
2. Add 1 g of soil sample in the first test tube, this tube is labeled as 10⁻¹ dilution and mix it well.
3. From the first tube (10⁻¹ dilution), take 1 ml of diluted sample and pour into the second test tube, this tube is labeled as 10⁻² dilution (Fig. 1).
4. Similar procedure of dilution is continued up to 10⁻⁸ dilution.
5. Prepare starch casein agar (150 ml) in 500 ml Erlenmeyer flask and sterilize the medium in autoclave at 121 °C, 15 min, 15 lbs. (see **Note 1**)
6. After the serial dilutions, 0.1 ml of last three middle dilutions (10⁻⁴, 10⁻⁵, 10⁻⁶) are separately plated on starch casein agar (SCA) using pour-plate method.
7. Incubate the inoculated plates at room temperature (28°C) for 5–10 days.
8. The *Streptomyces* colony forms a dry, powdery colonies, which have both aerial and substrate mycelium (Fig. 1).
9. The mycelium bears chains of spores at maturity.
10. The colonies with suspected *Streptomyces* morphology are sub-cultured in starch casein agar (SCA) medium.

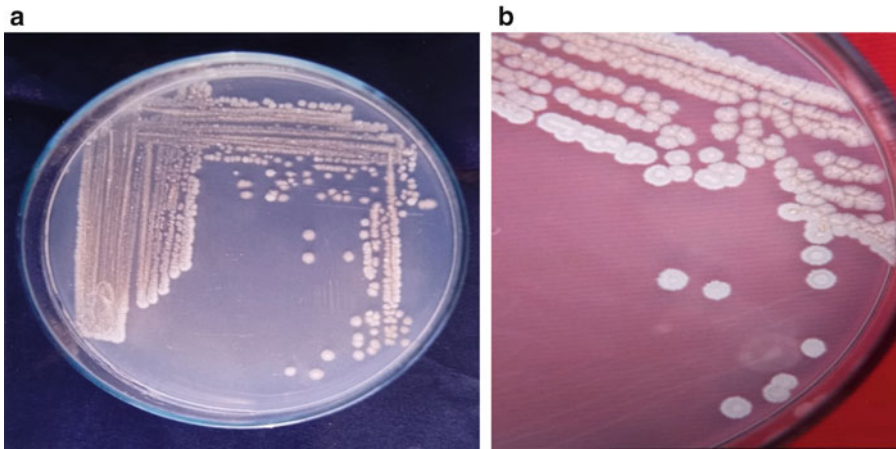


Fig. 1 (a) and (b) show colony morphology of *Streptomyces* isolates in starch casein agar (SCA)

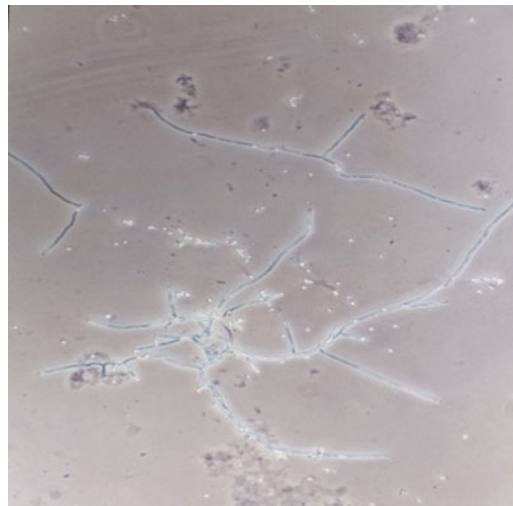


Fig. 2 Microscopic appearance of *Streptomyces* isolates in soil

11. The stock cultures are prepared for each isolate by transferring mycelium and spores from each of the purified isolates into cryotubes containing 1.5 ml 20% (w/v) glycerol stock and stored at -20°C .

Morphological observation by coverslip technique:

1. For observing the morphology, insert the coverslip in pure culture plates at the angle of 45° and incubate at 28°C for 5–7 days [5, 6].
2. Remove the coverslip after 5–7 days and place in clean glass slide by wet mount method.
3. Observe the slide under microscope and record the mycelia and spore shape and arrangements (Fig. 2).

4 Note

1. Incorporate the antibiotics nalidixic acid (25µg/ml), ketocozazole (100µg/ml) after the sterilization of starch casein agar (SCA) to prevent contamination of other microbes like bacteria and fungi.

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Chapter 17

Isolation and Identification of *Trichoderma* Spp. from Different Agricultural Samples

Harsh Mistry and Himanshu Bariya 

Abstract

Trichoderma can be isolated from soil, water, plant root, and decaying plant. *Trichoderma* plays key role in biocontrol of plant pathogens, plant growth promotion, and acts as a biofertilizer. In this chapter, easy and detailed steps have been described for the isolation and identification of *Trichoderma* spp. from different sources. *Trichoderma* can be isolated on Rose Bengal agar and *Trichoderma* selective agar medium. For species-level molecular identification, ITS amplification and sequencing as well as specific primers have been described.

Key words *Trichoderma* spp., *Trichoderma* selective agar medium, ITS region amplification, Sequencing, Species-specific primers

1 Introduction

Fungi from genus *Trichoderma* are very significant microorganisms as they possess various biopotentials like robust producers of antibiotics, industrial enzymes, and used as a biocontrol agent against numerous plant pathogens. They are abundantly found in soil, water, decaying plant tissues, and in the plant root system [1]. *Trichoderma*, a tremendous biocontrol agent which enhances the plant resistance against pathogen (e.g., *Aspergillus niger*, *Fusarium oxysporum*, and *Sclerotium rolfsii*), promotes plant growth by acting as a biofertilizer and also improves quality of soil. *Trichoderma harzianum* is reported for enhanced growth and yield of tomato and onion by secreting natural auxins like indole acetic acid (IAA), indole butyric acid (IBA), and gibberellic acid (GA3) and also increased efficiency of nutrient uptake in plants [2–4]. *Trichoderma* is also known for the stimulation of plant-defense response against different abiotic stress conditions (e.g., drought, water logging, mineral toxicity, extreme salinity, and temperatures). *Trichoderma harzianum* had shown its potential against drought when

inoculated with plants. Results showed higher enhancement of root and shoot length, chlorophyll, proline, and total soluble protein content in comparison of untreated sample [5–7]. Bulk harvesting of *Trichoderma* is very easy, cost-effective, and eco-friendly method and can be used directly for various application. In 2012, Dave et al. proposed economic method for powder formulation of *Trichoderma* which elicits enhanced defense response in *Brassica juncea* plant [8].

Additionally, *Trichoderma spp.* may intensify the soil quality. It has been reported that *Trichoderma harzianum* has augmented soil microbiological efficiency and β -glucosidase, urease, phosphatase, and dehydrogenase activities, further leading to saline soil rehabilitation [9]. A phosphorus and micronutrient deficiency in soil usually leads towards the limited growth of plants. *Trichoderma* plays an important role in the supply of phosphorus and micronutrients to plants and also in the productive use of phosphate fertilizers [10].

In addition, greater focus has recently been devoted to *Trichoderma* strains in bio-nanotechnology, specifically in the synthesis of various bioactive inorganic as well as metallic nanoparticles [11–14].

2 Materials

2.1 Media for *Trichoderma* Isolation

Trichoderma has been widely found in soil, lakes, air, wetlands, rivers, streams, wastewater, decaying plant parts, tropical and subtropical regions, wells, and elsewhere. Of course, the fungi are heterotrophic; they have the potential to get adapted in different biodiversity conditions. Fungi are usually contaminated in different commodities, including food and beverage goods, food storage facilities, and food processing facilities. Yeasts and molds can stimulate growth over a wide range of pH and temperatures in almost all forms of food, such as food ingredients and food processes. Various media compositions are given below (Tables 1 and 2) that can be used for the isolation of *Trichoderma*. Media must be autoclaved at 121 °C and 15 psi for 15 min, cooled down to 50 °C, and poured into petri dishes. Plate pouring and other necessary work should be done in laminar air flow, and work area should be sterilized with 70% ethanol prior to work to avoid microbial contamination.

2.2 Isolation of *Trichoderma* from Soil

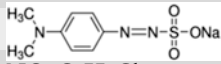
1. Soil sample.
2. Sterilized sample collection containers.
3. Conical flasks (several volumes).
4. 70% ethanol.
5. Sterile petri dish.

Table 1
Composition of Rose Bengal agar (RBA)

Sr. No.	Compound	Formula	Concentration (g/L)
1	Potassium dihydrogen phosphate	KH_2PO_4	1.0
2	Magnesium sulfate	$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.5
3	Peptone		5.0
4	Dextrose	$\text{C}_6\text{H}_{12}\text{O}_6$	10.0
5	Rose Bengal		0.35
6	Agar		15.0

Make up to 1000 mL with distilled water

Table 2
Composition of *Trichoderma* selective media (TSM)

Sr. No.	Compound	Formula	Concentration (g/L)
1	Dipotassium hydrogen orthophosphate	K_2HPO_4	0.9
2	Magnesium sulfate	$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.2
3	Potassium chloride	KCl	0.15
4	Ammonium nitrate	NH_4NO_3	1.0
5	Dextrose	$\text{C}_6\text{H}_{12}\text{O}_6$	3.0
6	Rose Bengal		0.15
7	Agar		20.0
8	Chloramphenicol	$\text{C}_{11}\text{H}_{12}\text{Cl}_2\text{N}_2\text{O}_5$	0.25
9	p-dimethylaminobenzenediazo sodium sulfonate		0.3
10	Pentachloronitrobenzene	$\text{NO}_2\text{C}_6\text{H}_4\text{Cl}$	0.2

Add 960 mL of distilled water

6. Glass spreader.
7. Wire loop.
8. Gloves.
9. Lab coat.
10. Shaker.
11. Autoclave.
12. BOD incubator ($28 \pm 1^\circ\text{C}$).

2.3 Isolation of *Trichoderma* from Water

1. Water sample.
2. Other materials as mentioned above in Subheading 2.2.

2.4 Isolation of *Trichoderma* from Plant Samples

1. Plant sample.
2. Sodium hypochlorite.
3. Other materials as mentioned above in Subheading 2.2.

2.5 Morphological Identification

1. Slide.
2. Distilled water.
3. Cotton Blue (Aniline Blue).
4. Phenol crystals ($C_6H_5O_4$).
5. Glycerol 100 mL.
6. Lactic acid ($CH_3CHOH COOH$).
7. 70% ethanol.
8. Microscope.

2.6 Molecular Identification (DNA Isolation, Amplification, and Sequencing)

1. *0.8% agarose gel for genomic DNA.*
 - (a) Add 0.8 g agarose powder to 100 mL $1 \times$ TAE buffer.
 - (b) Sprinkle agarose powder. Heat the flask until it gets digested.
 - (c) Cool down until it is not painful to touch and add 3 μ L of ethidium bromide.
 - (d) Cast the gel in casting assembly and allow it to get solidify.
2. *1.2% agarose gel for PCR amplified DNA.*
 - (a) Add 1.2 g agarose powder to 100 mL $1 \times$ TAE buffer.
 - (b) Follow the steps from 2–4 mentioned above.
3. Centrifuge.
4. Agarose gel electrophoresis apparatus.
5. UV transilluminator.
6. PCR.
7. Primers (Forward and reverse).
8. DNA sequencers and analyzers.
9. Micropipette.
10. GenElute™ PCR Clean-up kit.
11. BigDye® Terminator v 3.1 Cycle sequencing kit.

3 Method

3.1 Isolation of *Trichoderma* from Soil Sample

1. For the isolation of *Trichoderma*, soil samples can be taken from the depth of 15–20 cm.
2. Collect soil sample in sterile sample collection container by using sterile spatula.

TEN-FOLD SERIAL DILUTION

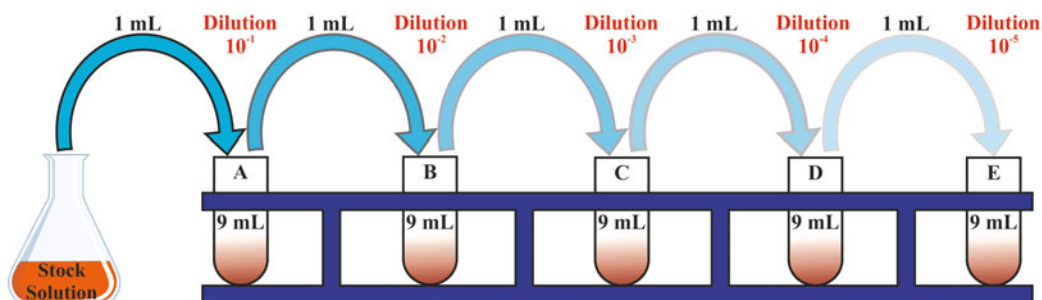


Fig. 1 Schematic representation of tenfold serial dilution of sample for *Trichoderma* isolation

3. Sample must be brought immediately to laboratory and stored at 4 °C until further use.
4. Suspend 1 g soil sample into 9 mL of sterile distilled water contained in a conical flask to gain 10^{-1} soil suspension.
5. Then gently shake soil suspension for 5–10 min.
6. Use sterile distilled water to obtain 10^{-2} , 10^{-3} , 10^{-4} , and 10^{-5} dilutions of soil suspension (Fig. 1).
7. Inoculate 100 μ L soil suspensions of each dilution into sterile petri plates containing agar media (RBA or TSM).
8. Spread the inoculum with the help of sterile glass spreader.
9. Incubate all the plates at 28 ± 1 °C in BOD incubator for 5–7 days.
10. Transfer *Trichoderma* colonies onto fresh PDA plates to obtain pure culture.

3.2 Isolation of *Trichoderma* from Water Sample

1. Water samples can be collected from ponds, rivers, and other water sources.
2. Prepare 10^{-1} to 10^{-5} dilutions of water suspension.
3. Inoculate 100 μ L water suspensions of each dilution into agar media (RBA or TSM) containing sterile petri plates.
4. Spread the inoculum with the help of sterile glass spreader.
5. Incubate all the plates at 28 ± 1 °C in BOD incubator for 5–7 days.
6. Transfer *Trichoderma* colonies onto fresh PDA plates to obtain pure culture.

3.3 Isolation of *Trichoderma* from Plant Root Sample

1. Choose healthy plants and dig up a small section of root using spade.
2. Store it in a labeled ziplock bag. Maintain the moisture inside the bag using spray bottle to mist the root.

3. Wash out bulk of soil from the root sample under tap water.
4. Cut 1 cm size of 5–6 pieces of root sections from the sample.
5. Perform surface sterilization by soaking root sections in 70% ethanol for 10 min.
6. Rinse the sections three times in sterilized distilled water and shake off the excess amount of water.
7. Place the root sections in media containing plates. Incubate all the plates at 28 ± 1 °C in BOD incubator for 5–7 days.
8. Transfer *Trichoderma* colonies onto fresh PDA plates to obtain pure culture.

4 Identification

4.1 Morphological Identification

1. Prepare a slide according to lactophenol cotton blue staining procedure and observe under $40\times$ microscope.
2. Fungi from *Trichoderma* genus have branched conidiophores that cluster into fascicles.
3. They show broad and straight or flexuous branches.
4. *Trichoderma* have conidial pigments which are either white or bright green in appearance (Fig. 2).

General Morphology of Trichoderma spp. on the PDA Media

1. Growth of the isolated *Trichoderma* colonies usually observe at 5 days at 28 ± 2 °C in darkness with one to two concentric rings near the inoculum zone with highly dense conidial production, with white conidia towards the center.
2. *Trichoderma* seems creamy in color and often folded or convoluted texture for the colony reverse.
3. No color diffusion or pigment is observed throughout the PDA plate.
4. Absence of sweet coconut odor and aerial mycelium (Table 3).

4.2 Molecular Identification

Accurate identification of *Trichoderma* at species level implies the use of molecular methods, precisely gene sequencing and analysis. Molecular identification of *Trichoderma* involves following steps:

1. Isolation of genomic DNA was from the fungal sample.
2. Amplification of the DNA fragment using high-fidelity PCR polymerase.
3. Sequencing of the PCR product bi-directionally.
4. Alignment analysis of the sequenced data to identify their closest neighbor genetics.

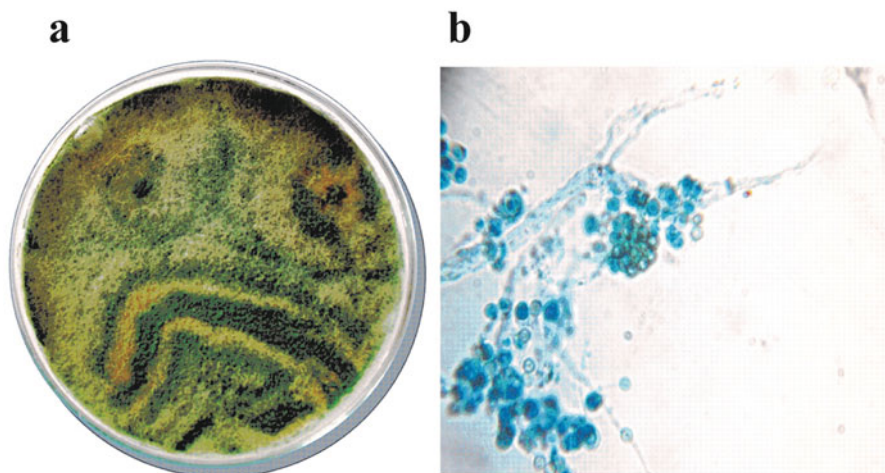


Fig. 2 (a) General growth pattern of *Trichoderma* spp. on PDA plate (b) Microscopic visualization of conidia of *Trichoderma* under 40 $\times$ lens

4.2.1 Isolation of Genomic DNA

1. Take 0.1 g of sample in a mortar and homogenize with 1 mL of extraction buffer and transfer the homogenate to a 2 mL microcentrifuge tube.
2. Add an equal volume of phenol:chloroform:isoamylalcohol (25:24:1) to the tubes and mix well by gently shaking the tubes.
3. Centrifuge the tubes at room temperature for 15 min at 16,464 $\times g$.
4. Collect the upper aqueous phase in a new tube and add an equal volume of Chloroform:isoamylalcohol (24:1) and mix.
5. Obtain upper aqueous phase by centrifuging at room temperature for 10 min at 16,464 $\times g$ and transfer to a new tube.
6. Precipitate the DNA from the solution by adding 0.1 volume of 3 M sodium acetate pH 7.0 and 0.7 volume of isopropanol for 15 min.
7. Centrifuge at 4 $^{\circ}\text{C}$ for 15 min at 16,464 $\times g$.
8. Wash the DNA pellet twice with 70% ethanol and then very briefly with 100% ethanol. Allow to air-dry the pellets.
9. Dissolve the DNA 30 μL TE (10 mM Tris-Cl, pH 8.0, 1 mM EDTA).
10. To remove RNA, add 5 μL of DNase-free RNase A (10 mg/mL) to the DNA.
11. Check the DNA on 0.8% agarose gel (Fig. 3).

Table 3
Macro and microscopic characteristics of various *Trichoderma* spp.

Character	<i>Trichoderma harzianum</i> ^a	<i>Trichoderma viride</i> ^a	<i>Trichoderma asperellum</i> ^b	<i>Trichoderma hamatum</i> ^b	<i>Trichoderma reesei</i> ^b
Macroscopic characteristics	Concentric rings	1–2 concentric rings	1–2 concentric rings	2 concentric rings	One or two concentric rings (old culture). One is at periphery and one is at inoculum point
Conidia	Green conidia	8.5–1–0.5 µm of dark-green conidia	Dense white conidial production	Pale yellow, greenish yellow to grayish green	Yellowish green in color
Coloration of the agar	Yellow to dark brown pigments	–	No color diffusion or pigment	No color diffusion or pigment	Intense diffusing yellow pigment
Mycelium structure	Initially smooth, watery white in color, and sparse	–	Aerial mycelium absent		Mycelium is not forming
Odor	Sweet coconut odor	Sweet coconut odor	Sweet coconut odor absent	Sweet coconut odor absent	No specific odor
Microscopic characteristics	Conidiophores	Highly branched and spread to the top in pyramidal fashion	Simple with unilateral branching or in pairs	Highly branched and arranged in symmetric order	Sparingly branches
Phialides	Flask shaped and typically short and	Arising from the main axis or in whorls at 2–3 at the tip of the	8 µm long, found at the tips of the primary,	Generally form from short lateral branches at the base of the	One or two phialides with cylindrical or slightly inflated

	broad with length of 4–6 µm	lateral branches or at the tip of the conidiophores	secondary, and tertiary branches	elongation and mostly ellipsoidal to ovoidal with an average length of 5–7 µm long, 3–4 µm wide at the widest point	shape. Average length is of 5–8 µm
Conidia	Globose to subglobose	Globose to subglobose	Globose to subglobose or ovoidal in the size of 3–4 µm	Smooth walled, ellipsoidal, and green in color	Smooth walled, pale green colored, oblong or ellipsoidal shape. Average length is of 3–5 µm
Pustules	Do not or poorly formed	0.5–1.0 mm, hemispherical, uniformly cottony	Do not form	–	–

^aObservations taken from our laboratory

^bObservations taken from book authored by Shafiquzzaman Siddiquee [15]

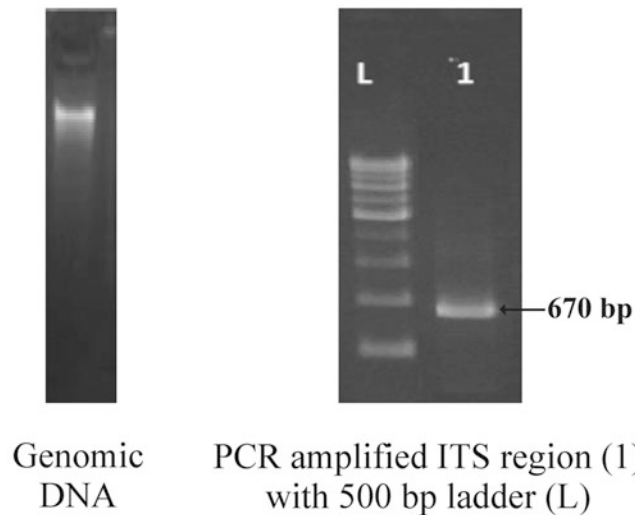


Fig. 3 Visualization of genomic DNA from fungi on 0.8% agarose gel and amplification of ITS region (670 bp) of fungi along with 500 bp ladder on 1.2% agarose gel

4.2.2 Amplification of the DNA Fragment

Polymerase chain reaction (PCR) is a fundamental laboratory technology for molecular biology. A particular DNA sequence can be directly approached with the PCR, and this sequence can be amplified to extremely high copy numbers. Amplification of DNA fragments involves following steps:

1. Prepare the PCR mixture as described in PCR mixture.
 2. Set the PCR amplification conditions as described in PCR amplification condition.
 3. Check PCR product on 1.2% agarose gel (Fig. 3).
- PCR mixture.*

Sr. No	Reactants	Quantity
1	DNA	1 μ L
2	ITS forward primer	400 ng
3	ITS reverse primer	400 ng
4	dNTPs (2.5 mM each)	4 μ L
5	10 $\times$ TaqDNA polymerase assay buffer	10 μ L
6	TaqDNA polymerase (3 U/mL)	1 μ L
7	Water	μ L
Total reaction volume		100 μ L

PCR amplification condition.

Sr. No	Reactions	Temperature (°C)	Time	Cycle
1	Initial denaturation	98	5 min	1
2	Denaturation	98	15 s	25
3	Annealing	62	30 s	
4	Synthesis	72	10 s	
5	Extension	72	5 min	1

Primers.

Sr. No	Primer name	Direction	Primer sequence (5'-3')	<i>Trichoderma</i> spp.	Reference
1	ITS 1	Forward	TCCGTAGGTGAACC TGC GC	Fungi (common)	[16]
	ITS 4	Reverse	TCCTCCGCTTA TTGATATGC		
2	Q01_3F	Forward	AAGCAAGGGGG TTGGCAAGTA	<i>T. Atroviride</i>	[17]
	Q01_3R	Reverse	GAGAAGGGGTTCCTC TGCAGAA		
3	QTh_8F	Forward	CATGGAAACACAGA CGGA	<i>T. harzianum</i> sensu stricto	[17]
	QTh_3R	Reverse	AGATAGAGA TGGAGAGAAAG		
4	HAM-450F	Forward	TTGACACGGTTCTA TAATTACCAA	<i>T. hamatum</i>	[18]
	HAM-450R	Reverse	TGACTTAAG TAAGCCGGG TCAAG		
Species-specific SCAR marker for identification of <i>Trichoderma</i> species					
5	Oligo 631	–	5'-ATCCGTACGC-3'	<i>T. harzianum</i> (1.6 kb)	[18]
6	OPA-5	–	5'-TGCCTGACTC-3'	<i>T. virens</i> (680 bp) <i>T. hamatum</i> (400 bp)	[18]

4.2.3 Sequencing of the PCR Product

Purification of Amplified PCR Products.

Carry out the purification of ITS gene amplified products using a GenElute™ PCR Clean-up kit (cat no. NA 1020-1kt) using following protocol:

Column Preparation:

1. Assemble GenElute plasmid mini spin column in 2.0 mL collection tube provided with kit.
2. Add 0.5 mL of the column preparation solution to the columns and centrifuge at $12,000 \times g$ for 30 s.
3. Add to 100 μ L of binding solution 20 μ L of PCR product. Transfer solution to the binding column after proper mixing.
4. Centrifuged the columns at $16,000 \times g$ for 1 min. Discard flow-through.

Column Washing:

1. Place binding column in a collection tube and add 0.5 mL of wash solution to the binding column. Centrifuge at maximum speed for 1 min. Repeat the step to remove the impurities.
2. Centrifuge at maximum speed for 3 min to dry the binding membrane and to prevent the alcohol contamination.

Elution:

1. Transfer the columns to the fresh 2 mL collection tube and apply 50 μ L of the elution solution to the center of each column.
2. Carry out DNA elution at maximum speed for 1 min. Store eluted DNA (PCR product) at -20°C .

Cycle Sequencing.

Use BigDye[®] Terminator v 3.1 Cycle sequencing kit for cycle sequencing. The BigDye Terminator v3.1 Cycle sequencing kit provides the required reagent components for the sequencing reaction in a ready reaction, premixed format.

Cycle sequencing reagent and concentration.

Sr. No	Reagents	Quantity
1	Terminator ready reaction mix v3.1	4 μ L
2	BigDye sequencing buffer	1 μ L
3	Template	15–300 ng
4	Primer	10 pM
5	Milli-Q water	Make up to 20 μ L

Cycle sequencing conditions.

Sr. No	Reactions	Temperature ($^{\circ}\text{C}$)	Time	Cycle
1	Incubation	96	1 min	–
2	Denaturation	96	10 s	25
3	Annealing	50	5 s	
4	Extension	60	4 min	
5	Hold	4	∞	–

Capillary electrophoresis parameters.

Polymer	Array	Run module	Mobility file
POP-7 TM polymer	50 cm	BDX_StdSeq50_ POP7xl	KB_3500_POP7xl_ BDTv3direct.Mob

4.2.4 Alignment Analysis of the Sequenced Data to Identify Their Closest Neighbor Genetics

1. Submit raw data obtained from sequencing to NCBI to obtain accession number.
2. Download the FASTA format from NCBI.
3. Process data in MEGA X to construct phylogenetic tree by using maximum likelihood method and Jukes-Cantor model.

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Chapter 18

Extraction, Isolation and Culturing of *Mycorrhizal* Spores from Rhizospheric Soil

Satish V. Patil, Bhavana V. Mohite, and Chandrashekhar D. Patil

Abstract

Agriculture is experiencing the time of innovation with soil and its biological interactions. *Mycorrhiza* is a fungus associated with plant roots. *Mycorrhiza* is an ancient living ubiquitous fungus in soil. A decade ago, J.L. Harley pronounced a sentence “Plants don’t have roots, they have mycorrhiza” in order to attract the attention of scientists towards plant-associated fungi in mutualistic association. *Mycorrhizae* increase the absorption of various nutrients, particularly phosphorus along with K, Si, Se, Zn, and Fe, and thus improve the crop productivity. The present chapter is focused on extraction, isolation, and culturing of *Mycorrhizal* fungi.

Key words Micronutrient, *Mycorrhiza*, Spores, Solubilization

1 Introduction

It is well known that the fungus plays various vital roles for mineral metabolism in soil and supplement to various plant. Bacteria and fungi are major contributors for P supplier to plant, besides these they promote nutrient availability, P, K, Si, Se, Zn, and Fe mobilizations through production of organic acids, metal chelators, protein, amino acids, and enzyme production (Fig. 1). *Mycorrhizae* are the nonpathogenic fungi which are associated with plant rhizosphere by mutualistic associations. They cause mild parasitism by invading roots for specific nutrients and provide various nutrients to plants. It is assumed that 90% of plants depend on *Mycorrhizal* supply of mineral nutrients especially phosphorus and iron (Fig. 2c). During some seasonal changes, they also provide nutrients like carbohydrate, sugar, and nitrogenous compounds. There are major two types as per the association, i.e., ecto *Mycorrhiza* and endo *Mycorrhizae*. Ecto *Mycorrhiza* means the fungus which is associated with external root surfaces and endo *Mycorrhiza* is associated with internal root cells of plant growing internally in plant

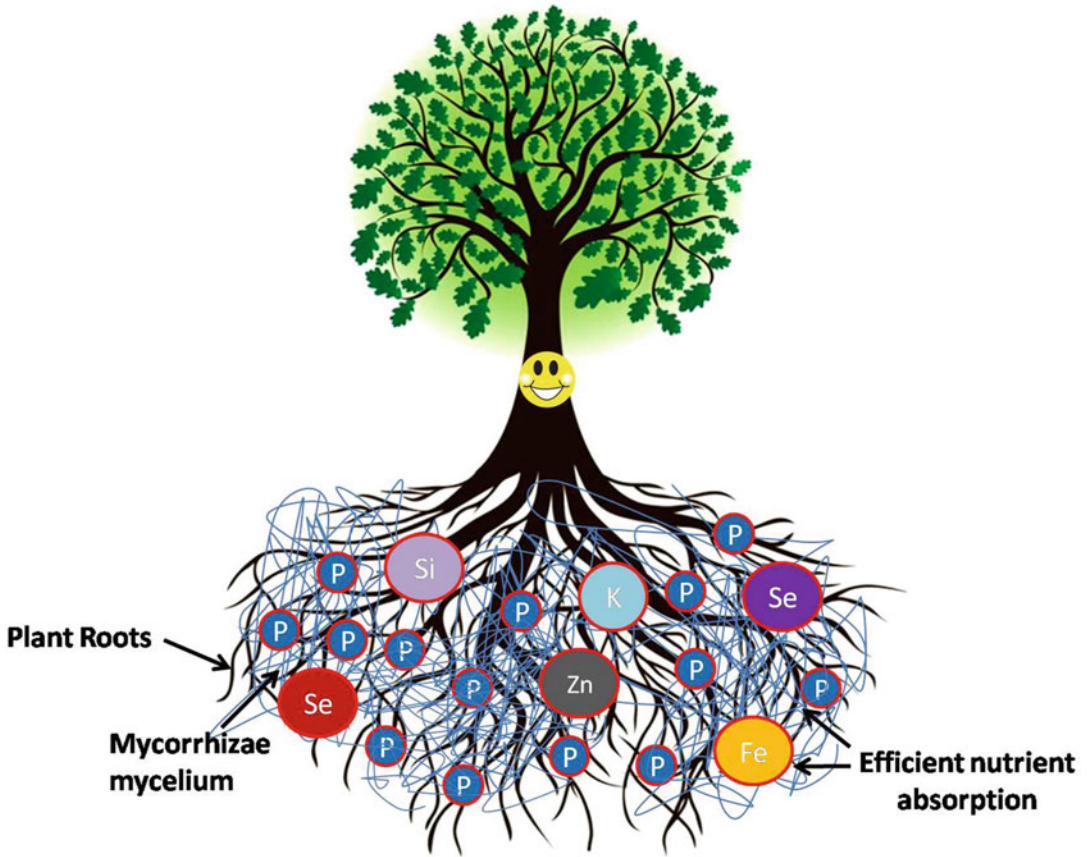


Fig. 1 *Mycorrhizal* plant roots mutualistic interaction

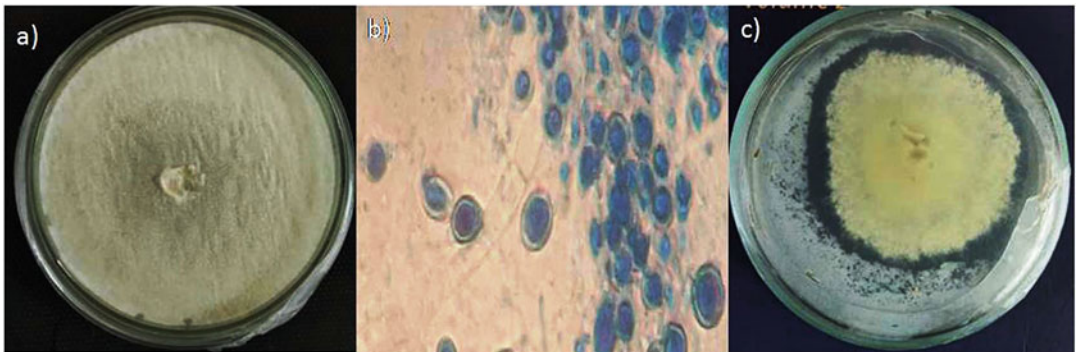


Fig. 2 (a) Growth of representative *Mycorrhizal* culture on agar plate (*Piriformospora indica*) (b) Microscopic observation of pear-shape *Mycorrhizal* spores (c) Zinc solubilization by *Piriformospora indica* (Dr. Satish V Patil Laboratory)

organs. Many times they are plant root specific, e.g., truffles *Terfezia boudieri* with oaks, *Larix* with larch plants, some *Rhizoctonia* with orchids. There is vast diversity of *Mycorrhizae* as per crop, plant, and area but generally the *Mycorrhizae* are reported by

detecting or extracting *Mycorrhizal* spores from soil and root samples. Although, there are some *Mycorrhizae* which do not produce always spore, such cultures may be identified by growing with plant tissue culture technique and other advance methods. Even if there are various techniques such as sucrose centrifugations [1] and adhesion floatation technique (AF) [2], capillarity adhesion method for spore extraction from soil and root are reported but wet sieving and decanting is the most practicable method for *Mycorrhizal* spore detection and isolation [3].

2 Principle of Assay

Extraction assay is based on principle that the *Mycorrhiza* are hydrophobic and light weight, and they float on water. These spores are separated by micro mesh sieves (250, 150, 53, and 45 μ m) and collected from the residue present on the sieves in petri plate and used for microscopic observation and cultivation (*see Note 4*).

3 Materials

- Rhizospheric soil/roots, rootlets, etc.
- Sterile containers for soil sample collection.
- Sterile tubes for serial dilution of soil samples.
- Staining dye 0.05% trypan blue in lactophenol.
- *Fragaria vesca* L., *Festuca ovina* L., and *Plectranthus* plantlets for cultivation of VAM.

4 Methods

4.1 Collection of Soil Samples and Extraction

1. Collect the 100 g of Rhizospheric or root associated soil samples/roots of specific plants at different locations.
2. Add 100 g soil sample in 1000 ml sterile water in glass container, mix vigorously for 2 min, and keep at 10 °C in refrigerator or in BOD incubator for 8–12 h.
3. After incubation, the supernatant slowly passes through the series of sieves 250, 150, 53, and 45/38 μ m (*see Note 4*).
4. Collect the residue present on each sieve separately in petri plates by washing sieves with sterile distilled water.
5. Observe the collected sieve wash under microscope.
6. For the plant root, dry the root material at 60 °C oven for 10–12 h, and grind the roots in grinder and soak the root

powder in sterile distilled water for 5 h, mix vigorously, and remove floating cellulosic material physically.

7. Allow the remaining water to pass through the sieves and follow above **steps 4** and **5**.
8. Collect the residue present on each sieve separately by washing with sterile water in petri plates.
9. Observe the spore morphology under microscope and identify the type of *Mycorrhizal* spore comparing with standard available cultures.
10. Using the dissecting microscope and micropipette, separate the *Mycorrhizal* spores and inoculate in sterile soil with plantlets of *Arabidopsis thaliana*, white clover *Trifolium repens*, Coleus (*Plectranthus scutellarioides*), in small pots, and allow to grow for 30 days (*see Note 3*).
11. Observe the root for *Mycorrhizal* colonization on root cells by root staining methods.
12. The spores collected/identified are inoculated with suitable coculturing plant and after 30 days observed *Mycorrhizal* association/spore, etc., and calculate its efficiency by following methods (*see Note 5*).
13. The culturable *Mycorrhiza*, i.e., *Piriformospora indica*, truffles *Terfezia boudieri* may be possible to culture on artificial medium in laboratory (*see Note 2*).

4.2 Staining and Determination of Percent of Mycorrhizal Association

1. Cut the roots in very small pieces, wash thoroughly under tap water and boil the pieces at 95 °C in 10% KOH for different time 10, 15, 20, and 25–30 min.
2. Then cool the material. Separate it, wash again with tap water then again treat with 1 N HCL for 5 min.
3. Stain the root pieces with 0.05% trypan blue lactophenol reagent. Mount the material on glass slide with fixing reagent, cover the material with coverslip, seal it by applying wax on corner, and observe under 40× (Fig. 2b) (*see Note 1*).
4. Measure the segment with spores and calculate the percentage of *Mycorrhizal* association by the following formula [4].

$$\% \text{ Mycorrhizal association} = \frac{\text{Number of Mycorrhiza associated segments}}{\text{Total Number of segments analyzed}} \times 100.$$

5 Notes

1. The fixative solution for staining the root is acetic acid; glycerol (1:1 V/V).
2. There are few *Mycorrhizae* spp. that are culturable on artificial medium, e.g., *Truffles. p indicus* (Fig. 2a). Other *Mycorrhizae*

fungi are only maintained with seedlings of various plants, e.g., *Trifolium repens*, *Coleus* (*Plectranthus scutellarioides*), *Arabidopsis thaliana*.

3. The plant specificity was reported for various *Mycorrhizae* sp., hence it should maintain on specific plantlets as per *Mycorrhizae* sp.
4. *Glomus* species spores generally retain in the 38/45µm sieve. It also catches the majority of spores including large *Gigaspora gigantea* and visible as bright greenish dots under microscope.
5. For better identification of spores, use spore plate photograph from diversity of arbuscular *Mycorrhizal* fungi associated with some medicinal plants in Western Ghats of Karnataka region, India or Distribution of arbuscular *Mycorrhizal* fungi (AMF) in Terceira and São Miguel Islands (Azores) Biodiversity Data Journal 8: e49759 doi: <https://doi.org/10.3897/BDJ.8.e49759>

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Isolation and Identification of *Metarhizium*

Tarun Kumar Patel

Abstract

The genus *Metarhizium* is genetically diverse that has been found to be pathogenic to over 200 species of insects. It can infect a number of economically important pests from termites to locusts. Commercial biopesticides formulations of *M. anisopliae* have been made for the control of several insect species. Members of *Metarhizium* are relatively slow growing but easily grown as pure culture on various culture mediums. Rapid growing soil fungi (from the genera like *Trichoderma*, *Mucor*, and *Rhizopus*) can overgrow on the *Metarhizium* colonies making their isolation rather more complex. Thus, the direct isolation of entomopathogenic fungi from soil usually depends on using a selective medium with the incorporation of an inhibitor to check the growth of undesirable organisms. The most accepted isolation method is the soil dilution plate method on a selective medium with the incorporation of some inhibitors and insect bait.

Key words *Metarhizium*, Entomopathogenic fungi, *Spodoptera litura*, *Metarhizium anisopliae*, Insect bait

1 Introduction

Various formulations of entomopathogenic fungi (EPF), *Metarhizium anisopliae* (Metchnikoff), Sorokin, and other species have emerged as excellent biological control agents for various insects [1–4]. *Metarhizium* shows the worldwide distribution and infects predominantly soil-inhabiting insects. *Metarhizium* belongs to the class Zygomycota and order Entomophthorales. The genus *Metarhizium* consists of more than 200 species with a broad host range, infecting five orders of insects [5]. All the members of this genus are filamentous fungi that reproduce by conidia which are formed on conidiophores [6]. They generally invade the host through the external integument; however, infection through the digestive tract may also be possible [7].

Table 1
Composition of oatmeal agar (OMA) medium

Components	Quantity (g/L)
Oatmeal :	20.0
CTAB :	0.6
Chloramphenicol :	0.5
Agar :	15.0

Table 2
Composition of Sabouraud’s dextrose agar yeast extract (SDAY) medium

Components	Quantity (g/L)
Dextrose :	40.0
Yeast extract :	10.0
Peptone :	10.0
Agar :	15.0

2 Materials

**2.1 Isolation from
Soil Samples (see
Note 1)**

- Soil.
- Litter.
- Insect cadavers.
- Culture media (Oatmeal agar [8] (Table 1); Sabouraud’s dextrose agar yeast extract (Table 2)).

**2.2 Rearing
of *Spodoptera litura*
(*Lepidoptera Fab*)**

- Adult moth.
- Humidity chamber.
- Cotton swab.
- Castor leaves.
- Incubator.
- Sodium hypochlorite.
- Ethanol.
- Plastic box.

3 Methods

3.1 Soil Dilution Plate Method/Culture Plate Method (see Notes 2–4)

1. Isolation of *Metarhizium* from soil samples can be carried out using oatmeal agar (OMA) medium containing cetyl trimethyl ammonium bromide (CTAB) (Table 1) as described earlier [8] for preferential selection of *Metarhizium* and other EPFs.
2. Serially dilute the soil suspension (0.1 mL) and spread over the OMA plates aseptically.
3. Leave the plate partially open in an aseptic condition for a while to evaporate the excess water from the medium surface.
4. Incubate the plates in an inverted position at 25 ± 2 °C for 10–20 days.
5. On the appearance of fungal colonies with conidia, purify the colonies through pure culture technique and maintain on SDAY slants and also as glycerol stocks at 4 °C for further uses.

3.2 Rearing of *Spodoptera litura* (*Lepidoptera Fab*)

1. Transfer newly emerged adults (moth) of *Spodoptera litura* in a fabricated cage covered with net (mesh size 1 mm).
2. Surface-sterilize the cage with ethanol (70%, v/v) and UV irradiation for the complete elimination of contaminating microorganisms.
3. Place the cage in an incubator at 25 ± 2 °C with 12 L:12 D h photoperiod; relative humidity at $85 \pm 2\%$.
4. Feed the moths on honey:water (1:4) solution soaked in a cotton swab and place in a small petri dish.
5. Maintain two sets of plates for 12 adults (6 females and 6 males).
6. Surface-sterilize the stalked young castor (*Ricinus communis* L.) leaves of suitable size in a 100 mL flask containing sterile distilled water (for maintaining turgidity of leaves) inside the cage which serve as an oviposition site for the moths.
7. Change the leaves with fresh leaves at an interval of 24 h along with the feed. Examine the foliage every day and collect the eggs [9].
8. Collect the eggs from the walls of the cage with the help of a fine paintbrush. Before scrapping, remove the hair-like scales from the egg mass surface with a smooth small brush.
9. Transfer the egg masses to ethanol washed, UV-sterilized small plastic boxes, each containing not more than 15 egg mass.
10. Incubate the boxes in an incubator as abovementioned conditions for hatching.
11. After hatching, transfer about 50 first instar larvae to fresh sterile plastic containers with the help of soft paint brush.



Fig. 1 Larvae of *Spodoptera litura* feeding on surface-sterilized stalked young castor (*Ricinus communis* L.) leaves

Place one or two moist blotting paper in the bottom of containers and keep the boxes in the incubator.

12. Feed the early stages of larvae (first and second instar) with tender castor leaves. Prior to use the surface, sterilize the leaves with sodium hypochlorite (0.5%, v/v) and wash with sterile distilled water.
13. On the other hand, feed the third to sixth instar larvae on mature castor leaves. After third instar larvae, change the castor leaves twice a day (Fig. 1).
14. Distribute the sixth instar stage larvae into plastic boxes with not more than 5 larvae per box to avoid crowding.
15. In natural conditions, *S. litura* pupates inside the soil. However, in the laboratory, the sixth instar larvae cover with surface-sterilized castor leaves and tissue paper cramps to mimic natural conditions. The developing/newly developed pupae should not be disturbed (Fig. 2).
16. After 2 days, surface-sterilize the pupae, when turned brown, with sodium hypochlorite solution (0.5%, v/v) and leave it for being metamorphosed into adult moths. Different stages of life cycle of *S. litura* are shown in Fig. 3.

3.3 Insect Bait Method [10] (see Note 5)

1. Homogenize and air-dry the collected soil samples (softly grind the soil sample only if required) to avoid infections by entomopathogenic nematodes.
2. Remoisten the soil samples with required volume of sterile distilled water to maintain an appropriate level of humidity.

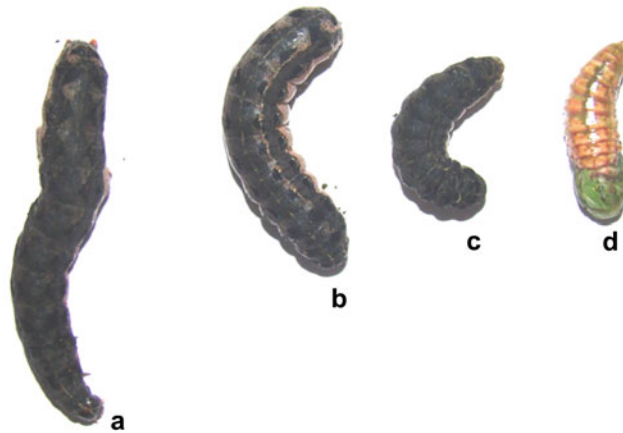


Fig. 2 Metamorphosis from larvae to pupae in *S. litura*. (a) fully grown sixth instar larvae, (b) feeding and other activities of larvae, (c) larvae gradually shrinks and reduces body mass, (d) newly metamorphosed pupae

3. Weigh about 100 g of moist soil sample in a sterile plastic container with perforation on the lid for aeration.
4. Perform isolation of *Metarhizium* by transferring the insect (fourth instar *S. litura* larvae are used as bait) in to the soil sample.
5. Prior to inoculation, surface-sterilize the larvae with sodium hypochlorite solution (0.5–%, v/v) for a minute followed by two changes of sterile distilled water to remove any traces of sodium hypochlorite (*see* **Notes 6–8**).
6. Subsequently dry the larvae by blotting with UV-irradiated tissue paper and then transfer the surface-sterilized larvae onto the soil sample in a sterile jar with perforated lid.
7. Feed the larvae using surface-sterilized fresh castor leaves.
8. Incubate the containers at $25 \pm 2^\circ\text{C}$ in the dark in incubator.
9. Invert the individual containers for proper mixing of soil with insects every day for a week.
10. Observe the boxes for every day for the dead larvae.
11. Collect the collected dead larvae immediately, surface-sterilize with sodium hypochlorite (0.5–%, v/v), and wash with two changes of sterile distilled water.
12. Place the surface-sterilized dead larvae aseptically over 3–4 layers of moist blotting papers in petri plates and incubate it for the growth of fungi, and further conidiation.
13. Observe for the appearance of fungal growth and conidiation over the dead larvae (Fig. 4), and transfer the insect cadaver with mycelia and conidiation to the selective medium (OMA with CTAB) for the further confirmation or can be directly

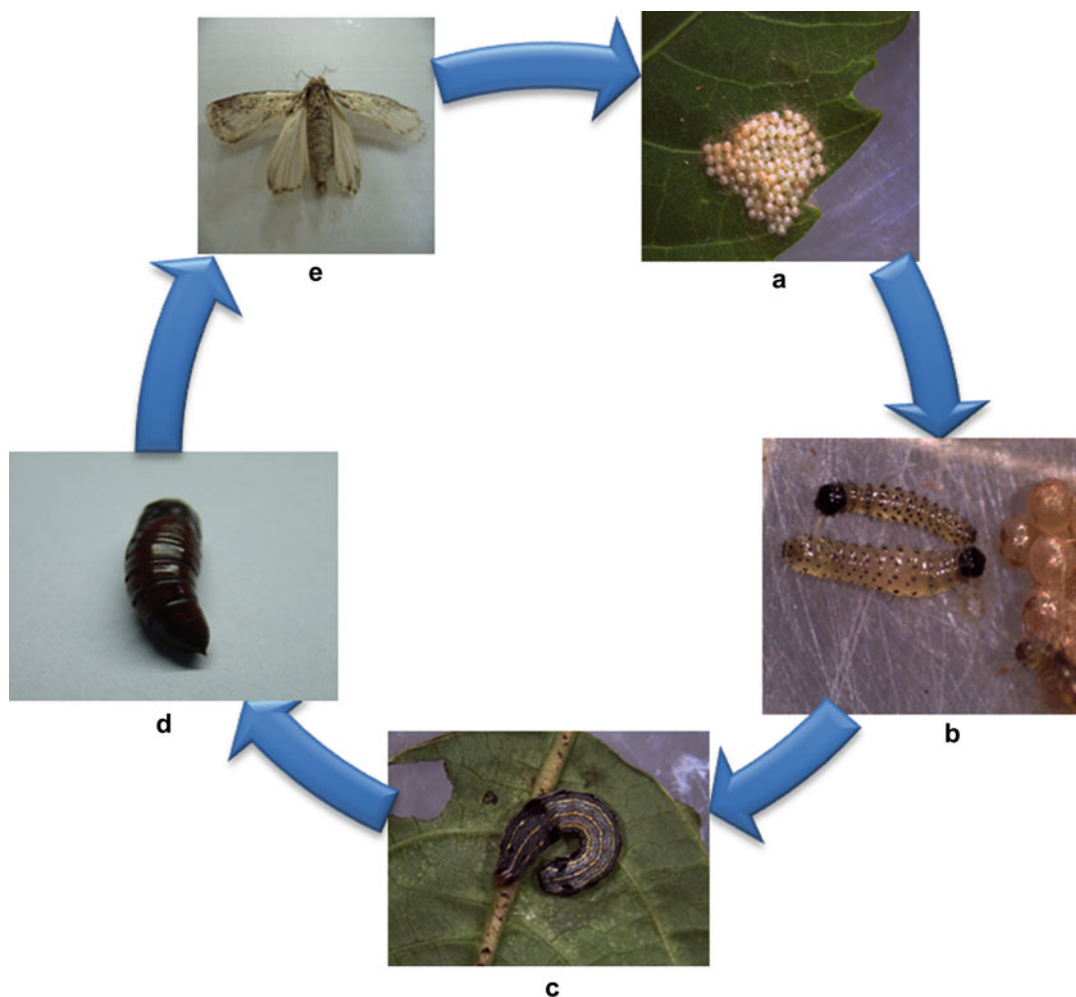


Fig. 3 Stages of life cycle of *S. litura* completed in laboratory condition. (a) egg mass laid on castor leaf, (b) newly emerged first instar larvae, (c) a well grown fourth instar larvae, (d) pupae of the insect, (e) adult emerged from pupae

cultured on SDAY for further macroscopic and microscopic identification.

14. Pure cultures of fungal isolates can be stored on Sabouraud's dextrose agar yeast extract (SDAY) (Table 2) slants as well as in glycerol stocks and preserve at 4 °C for future use (*see Note 9*).

3.4 Microscopic Characterization

1. Observe fungal isolates microscopically after staining them with lactophenol cotton blue (LPCB) to increase the contrast and improve the visibility of hyaline mycelium [11].
2. Place a single drop of LPCB over a clean glass slide and scrap mycelia bearing conidiophores from the freshly cultured plates with the help of a sterile needle to the stained area of slide.

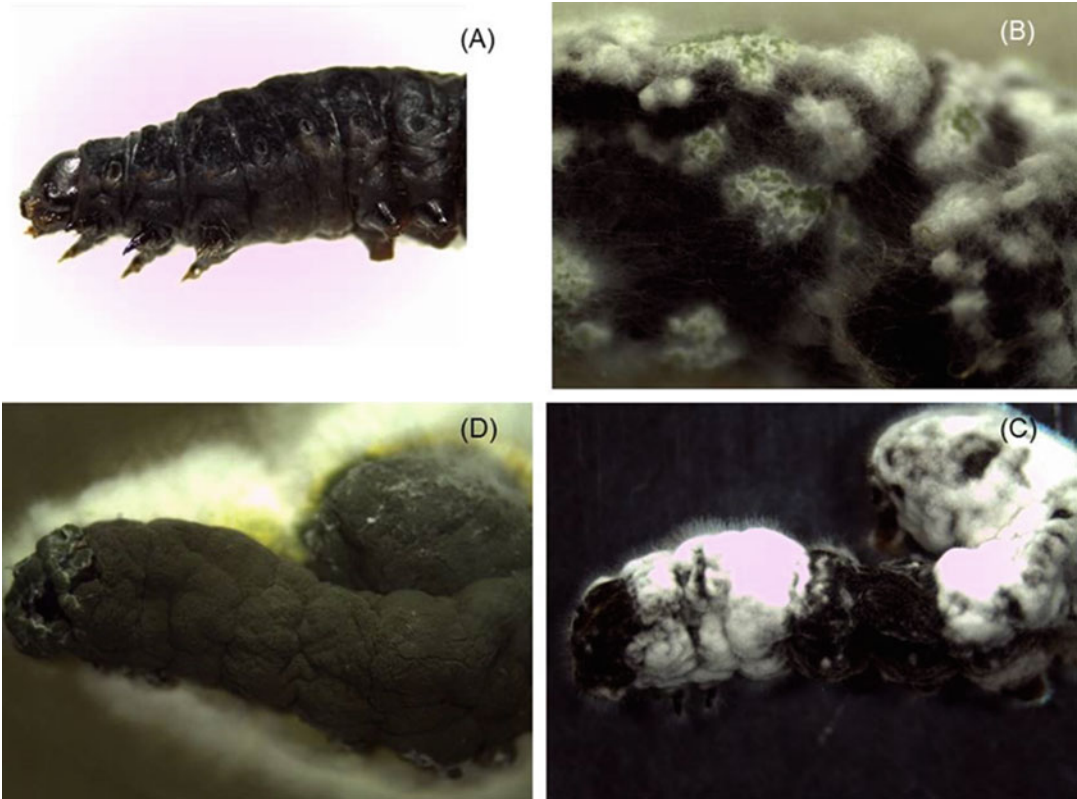


Fig. 4 Different stages of dead larvae of *Spodoptera litura* showing mycelia growth and conidiation due to *Metarhizium*. **(A)** recent dead larvae of *Spodoptera litura* **(B)** after 5 days growth of *Metarhizium* can be seen all over the surface of dead larvae **(C)** after 10 days thick mycelial growth can be observed **(D)** after 15 days fungal mycelia is covered with conidia.

3. Cover the mycelia with a thin glass coverslip.
4. Remove air bubbles trapped between the slide and coverslip by gentle heating and wiping the excess of stain with lint-free tissue paper moistened with ethyl alcohol.
5. Observe the morphological features of mycelia, conidia, conidiogenous cell, and conidiophores, etc., under the microscope (Axio scope.A1, Zeiss).

3.5 Molecular Identification

Molecular identification of selected isolates can be performed through sequencing of the conserved region of 18S rDNA and ITS region including ITS1, 5.8S, and ITS2 region, as detailed in the following subsections.

3.5.1 Isolation of Genomic DNA

1. Extract genomic DNA (gDNA) from fungal isolates grown in liquid culture, using phenol:chloroform method [12].
2. Check the quality of gDNA through agarose gel electrophoresis (Agar 0.8%, w/v) in TAE buffer [13] and visualize over UV transilluminator (Model no. 2000, Bio-Rad, USA).

3. Quantify the gDNA using Nanodrop spectrophotometer (Thermo Scientific, USA) at A260/A280 nm.

3.5.2 Amplification of ITS Region and Sequencing

1. Amplify the ITS region of *Metarhizium* genome using universal ITS region primers (forward primer ITS1, 5'TCCGTAGGTGAACCTGCGG 3' and reverse primer ITS4, 5'TCCTCCGCTTATTGATAT3') as per standard procedure.
2. Purify the PCR product (Qiagen GmbH, Germany), and sequence using forward primer by ABI-3500 DNA sequencer (Applied Biosystems, Switzerland).

3.5.3 Sequence Similarity Search and Submission

1. Subject the obtained sequences to National Center for Biotechnology Information (NCBI), for BLAST analysis using the BLASTn program for sequence similarity search from the available data repository [14–16].
2. Identify the fungal strain based on the highest sequence similarity resulted from BLAST analysis.

4 Notes

1. Samples should be collected before or after the rainy season (preferably in spring season) to reduce the cross-contamination by undesired fungi and bacteria [21].
2. Inhibition of contaminating fast-growing saprophytic fungi is more problematic in case of *Metarhizium* (and other EPFs) than bacteria [17, 19].
3. Addition of dodine in oatmeal agar medium improves the isolation of EPF. In addition, media amended with crystal violet enhances the contrast of fungal colonies. However, 460 mg/L dodine and 380 mg/L of the fungicide benomyl are found to be suitable for effective isolation of *M. anisopliae* [18]. Whereas some isolates of *M. anisopliae* are inhibited at 300 mg/L of dodine, and a modified formulation with a reduced concentration of dodine (10 mg/L) and 500 mg/L of cycloheximide led to improved recovery of *Metarhizium* from soil [19, 22].
4. Perform all the experiments under highly aseptic condition [9].
5. The insect bait method can be performed with any other insect-like *Galleria mellonella* or any locally available insect or the target insect for the isolation of species-specific virulent strain isolation [4–6].
6. Larvae and castor leaves should be properly sterilized using a sodium hypochlorite solution to ensure the larvae free from infections by undesirable microorganisms [9].

7. The concentration of sodium hypochlorite solution for surface sterilization should be optimally 0.5–1%, v/v or maybe higher, and it depends on the level of contamination [9].
8. The larvae rearing boxes should be cleaned every day to avoid contamination by saprophytic fungi and bacteria on insect feces [9].
9. In the insect bait method, the larvae which show stiffness with time should be chosen for further mycelial growth and conidiation as it is the sign of infection by *Metarhizium*, whereas the larvae with the soft body should be discarded immediately as the infectious agent might be saprophytic fungi or bacteria [20].

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Chapter 20

Isolation and Identification of Bacteriophage for Biocontrol

Mitesh Dwivedi

Abstract

Many plant diseases including bacterial wilt have no promising control strategy to eradicate the bacterial pathogens from soil or water, or to cure infected plants. Virulent bacteriophages which are specific for their hosts can be employed as phage therapy for such plant diseases to kill the bacterial pathogens. Such bacteriophages can be isolated from the environmental samples (soil or water) and their biocontrol activity can be assessed. The current chapter delivers the protocol for isolation, purification, and identification of such bacteriophages from water sample, and assessment of their biocontrol activity on host plants.

Key words Bacteriophage, Biocontrol, Water sample, Purification of phage, Plaque assay, Enrichment detection assay, Double-layer agar technique

1 Introduction

Viruses that infect bacteria are known as bacteriophages. They eat bacterial cells and generate a clearing, or plaque, on a lawn of susceptible bacteria [1]. The bacteria are killed by lysis as newly produced phages are released from the damaged cells. Like all viruses, bacteriophages consist of nucleic acid (RNA or DNA) surrounded by a protein coat, or capsid. Unlike some plant and animal viruses, bacteriophages are not enveloped. Some phages have elaborate structures for attaching to the bacterial surface and injecting nucleic acid into the cytoplasm.

The plaque assay is originally a virological assay employed to count and measure the infectivity level of the bacteriophages [2]. This assay is the most widely used technique (double-layer agar technique) for the isolation of virus and its purification, and to optimize the viral titers. The assay is based on the fact that each plaque on a lawn of bacteria (concurrent monolayer culture cells), although it contains 10^6 to 10^7 virions along with bacterial debris, represents a single infecting phage that entered one cell at the start of the culture. The infection then spreads as the viruses reproduced

and cells lysed, eventually forming a visible plaque. The titer of a phage suspension, therefore, is determined by counting the number of plaques that form from a given volume of suspension. Phage titer is expressed as plaque forming units (PFU) per milliliter (mL).

The chapter deals with the isolation, purification, and screening of lytic phages which can serve as biocontrol agent against plant pathogens. For example, *Ralstonia solanacearum* is a causative agent of bacterial wilt disease, which is one of the most important vascular plant diseases [3]. In spite of the economic importance of the disease on various crops in many tropical and subtropical areas, disease management is highly limited. There are no effective chemicals available to eradicate the bacterial pathogens from soil or water, nor to cure infected plants. Hence, the lytic (virulent) phages can be isolated from environmental water samples which specifically infect *R. solanacearum* strains, and some of the closely related pathogenic species such as *Ralstonia pseudosolanacearum*, without affecting nontarget environmental bacteria. These lytic phages are able to lyse the pathogen populations within a wide range of conditions comprising environmental values of water temperatures, pH, salinity, and lack of aeration found in storage tanks [4]. These phages belong to the *Podoviridae* family and are members of the *T7-like virus* genus.

The current chapter delivers the protocols for the isolation, purification, identification, and screening of lytic phages possessing biocontrol activity. These protocols are useful and can be employed for exploring any specific lytic phages as biocontrol agent for the prevention and/or biocontrol of the various bacterial diseases caused by plant pathogens such as *R. solanacearum*.

2 Materials

2.1 Isolation of Bacteriophages

Environmental sample (water sample, soil sample, etc., from the disease affected areas), 24-h nutrient broth cultures of specific plant pathogen bacterium, e.g., *Ralstonia solanacearum* strain IVIA-1602.1, one (125 mL) Erlenmeyer flask containing 40 mL of river water sample, 1× nutrient broth, 10× nutrient broth, warmed nutrient agar plates, tubes containing 3 mL each of warm, top agar (one per plate), 10 mM phosphate-buffered saline (PBS) pH 7.2, yeast extract peptone glucose agar (YPGA) plates, modified Wilbrink broth (MWB), 28 °C incubator with shaker platform, water bath at 28 °C, water bath at 45 °C, 5 mL pipettes/pipettor, 15 mL conical centrifuge tube, 1.5 mL microfuge tubes for preparing dilutions, 1.0 mL serological pipettes, pipettor, micropipettes, microtips (100–1000 mL), laboratory marker, and labels.

- *Composition of yeast extract peptone glucose agar (YPGA):*

Ingredients	g/L
Bacto peptone	5.0
Yeast extract	5.0
Glucose	10.0
Agar	15.0
Distilled water	1 L
Adjust pH to 7.0–7.2	

- *Modified Wilbrink broth (MWB):*

Ingredients	g/L
Sucrose	10.0
Proteose peptone	5.0
K ₂ HPO ₄	0.5
MgSO ₄ ·7H ₂ O	0.25
NaNO ₃	0.25
Distilled water	1 L
Adjust pH to 7.0–7.2	

2.2 Purification of Bacteriophages

Polyethylene glycol (PEG), 0.45 µm pore-size membrane filter, SM buffer (composition is given below), EGTA (at final concentration of 1 mM), and cooling centrifuge.

- *Composition of SM Buffer:*

Tris–HCl (pH 7.5)	50 mM
NaCl	100 mM
MgSO ₄	10 mM
Gelatin	0.01%

2.3 Identification of Bacteriophage

2% phosphotungstic acid (PTA) (pH 7.4), Formvar–carbon coated copper grid, transmission electron microscope (TEM; JEM-2010; JEOL, Tokyo, Japan), DNase and RNase (1 mg/mL), polyethylene glycol (PEG) 8000 1:10 (w/v), 20% SDS, proteinase K (20 mg/mL), phenol, chloroform, and isoamyl alcohol mixture at 25:24:1 (v/v), ethanol (100 and 70%), nuclease-free water (NFW), cooling centrifuge, UV spectrophotometer, thermocycler (BioRad), 2100 Bioanalyzer and Illumina sequencer HiSeq 2500/1500.

2.4 Screening of Isolated Phages for Biocontrol Activity

2.4.1 Biocontrol of Phages in Environmental Water

R. solanacearum strain IVIA-1602.1, 0.22-mm membrane filter, river water samples, isolated bacteriophages, PBS, YPGA medium, and incubator cum shaker.

2.4.2 Biocontrol in Host Plants by Watering

Tomato plants or plants of interest, *R. solanacearum* strain IVIA-1602.1, isolated bacteriophages, PBS, Biosafety Level 3 (BSL3), and semi-selective medium South Africa (SMSA) agar.

- *Composition of semi-selective medium South Africa (SMSA) agar:*
 - Prepare 1 L of TTC (or TZC) medium (except substitute glycerol for the glucose):

Ingredients	Per liter
Casamino acid (casein hydrolysate)	1 g
Peptone	10 g
Glycerol	5 mL
For solid media (plates) add agar	17 g
Adjust pH to 6.5–7.0	
Autoclave at 121 °C for 20 min	

- After autoclaving, cool the medium to 55 °C and add 5 mL of a 1% stock solution of 2, 3, 5-triphenyl tetrazolium chloride. (The stock can be filter-sterilized or autoclaved for 5 min at 121 °C, and stored at 4 °C or frozen.)
- On solid medium, colonies of *R. solanacearum* usually are visible after 2–5 days of incubation at 28 °C. Typical bacterial colonies appear fluidal, irregular in shape, and white with pink centers.

3 Methods

3.1 Isolation and Purification of Bacteriophages

3.1.1 Sample Collection

1. Collect water samples from rivers located in the vicinity of specific plant fields (such as tomato or potato) formerly affected by bacterial wilt.
2. Use sterile bottles for the collection of water samples and close them immediately.
3. After sealing the bottles cap, transport the water sample at 2–8 °C to the laboratory.

3.1.2 Enrichment Detection Assay

Enrichment detection assay is used for isolation of bacteriophages. For example, *R. solanacearum* lytic phages can be isolated according to protocol given by Alvarez et al. [5], with minor modifications.

Amplification of Bacterial Viruses

1. Inoculate a 50 mL of $1\times$ nutrient broth with *R. solanacearum* or the specific plant bacterium strain for overnight growth at 28°C with shaking.
2. The suspension of *R. solanacearum* strain IVIA-1602.1 (O.D. at 600 nm = 1) should contain about 10^9 colony-forming units (CFU/mL) in a modified Wilbrink broth (MWB) or nutrient broth [6].
3. Filter the 40 mL river water sample using the $0.22\ \mu\text{m}$ sterile filter in flask.
4. Pipette 5 mL of $10\times$ nutrient broth into the flask containing 40 mL of river water sample.
5. Inoculate this flask with 5 mL of an overnight culture of *R. solanacearum*. Bacterial suspensions without filtered water serve as negative controls.
6. Also, inoculate a separate flask containing 45 mL of $1\times$ nutrient broth with 5 mL of an overnight culture of *R. solanacearum*.
7. Incubate both cultures at 28°C , shaking (200 rpm) for 24 h.

Bacteriophage Isolation and Plating

1. Inoculate 10 mL of $1\times$ nutrient broth with *R. solanacearum* (overnight grown culture) at 28°C with shaking.
2. Transfer 10 mL of the river water-bacteria-bacteriophage culture into a centrifuge tube, and centrifuge the sample at $448\times g$ for 5 min. Most of the remaining cells will be pelleted. The supernatant contains bacteriophage.
3. Prepare a series of microfuge tubes for making serial tenfold dilutions of the bacteriophage suspension (performing the same dilution repeatedly in series is called serial dilution (Fig. 1)). Label six tubes 1–6. Into each tube, pipette 0.9 mL of sterile PBS (pH 7.2).
4. Perform serial dilutions: Transfer 0.1 mL of phage suspension (that has been mixed well) into tube 1, and mix. Using the same pipette, transfer 0.1 mL of the sample from tube 1 into tube 2, and mix. Repeat this process, transferring 0.1 mL from tube 2 to tube 3, and so on, mixing each time, as shown in Fig. 1. Store the remaining phage suspension in the refrigerator.
5. Distribute 0.5 mL of log-phase *R. solanacearum* into each of six microfuge tubes, labeled 1–6.

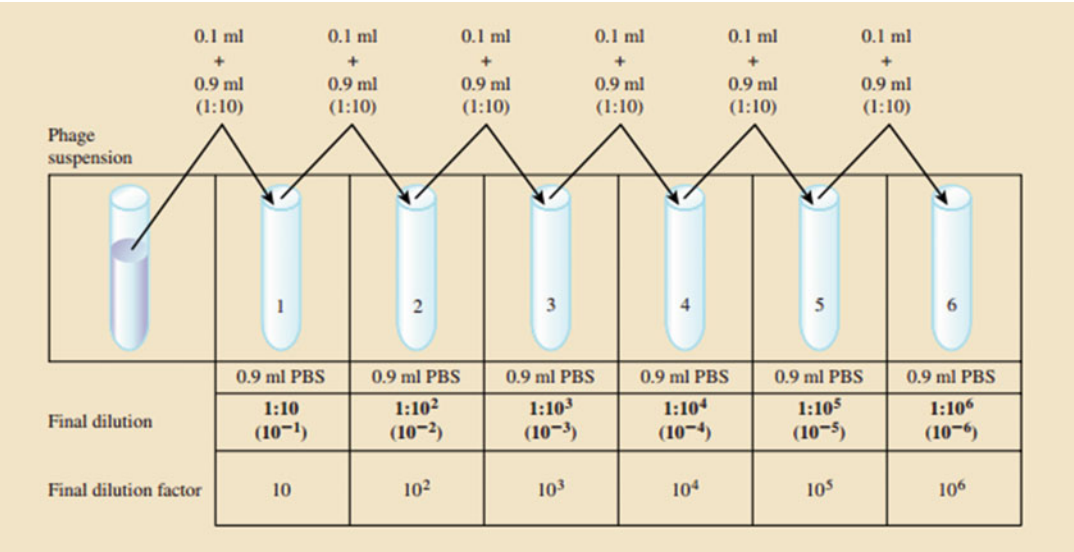


Fig. 1 Serial dilutions of bacteriophage suspension. First, pipette 0.9 mL of PBS (diluent) into each dilution tube (numbered 1–6). Then, transfer 0.1 mL of phage suspension in series, mixing each time

6. To each tube of bacteria, add 0.1 mL of the corresponding phage dilution (0.1 mL of dilution 6 to cell tube 6, and so forth). Cap the tubes, and mix gently by inverting them.
7. Incubate the tubes at 28 °C for 10 min to allow the phage to adsorb (attach) to the bacteria. This is the cell–phage mix.
8. In the meantime, label six warm, dry, nutrient agar plates 1–6 (one for each infection) or YPGA plates (for *R. solanacearum*). Write on the bottom plate along the plate edge.
9. According to a standard surface plating method [7], add the contents of cell-phage tube 1 to a vial containing 3 mL of top agar (molten, at 45 °C). Quickly cap the tube, and mix it by gently inverting it three times. Quickly pour the mixture onto plate 1. Tip the plate slightly to spread the top agar. Push the plate aside, but do not pick it up until the agar solidifies.
10. Repeat **step 9** for each of the remaining five samples, 2–6.
11. Allow the plates to cool without being disturbed for approximately 10 min. When the top agar has solidified, incubate the plates, inverted, at 28 °C for 24 h.

Examination of
Bacteriophage Plates and
Phage Titer Determination

1. Record the number of plaques on each plate in the laboratory report and count the phage titer.
2. See Fig. 2 for the plaques formed. (Note: If the plate is very crowded, it may be easier to count; if the plate is divided in quarters or eighths and then multiply the count by 4 or 8, respectively.)
3. Record the results in the following table.

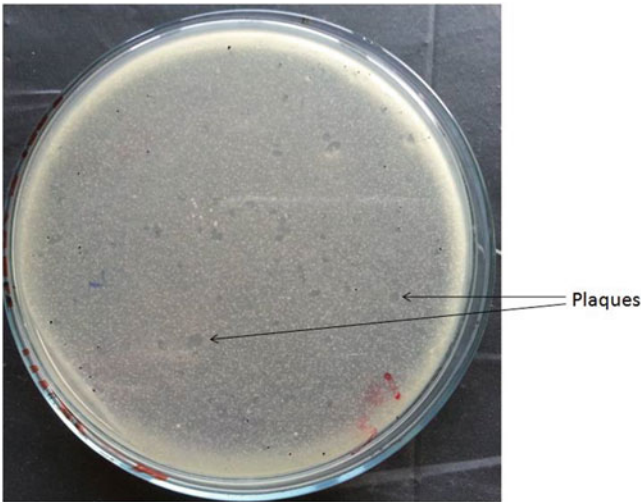


Fig. 2 Representative plate for plaque formation (plaque assay: double-layer agar technique)

Plate no.	Plaques per plate	Dilution factor	Volume of phage plated (mL)	Phage titer calculation: $\frac{(\text{number of plaques}) \times (\text{DF})}{\text{Volume plated (mL)}}$	Average phage titer: Plaque-forming units (PFU) per mL
1.					
2.					
3.					
4.					
5.					
6.					

3.2 Purification of Bacteriophage

Further, the phage can be purified by the standard protocol of polyethylene glycol (PEG) precipitation [8, 9].

1. Select and collect the plaques from the plates and centrifuge it at $8000 \times g$ for 20 min at 4 °C.
2. Filter the supernatant using a 0.45 μm pore-size membrane filter and precipitate phage particles by centrifugation at $40,000 \times g$ for 1 h at 4 °C and then suspend it in SM buffer.
3. Store the purified phages at 4 °C until needed. [Note: To increase the phage recovery, add EGTA (at final concentration of 1 mM) to the phage infected culture at 6–9 h postinfection.]
4. In addition, verify the lytic activity of the purified bacteriophages against the plant pathogen, e.g., *R. solanacearum* liquid cultures in MWB or in solid general media.

3.3 Identification of Bacteriophage

3.3.1 By Transmission Electron Microscopy (TEM)

The transmission electron microscopy after negative staining with a solution of 2% phosphotungstic acid (PTA) is used to identify the morphology of the phage as mentioned by Bae et al. [10]

1. Take the drops of purified phage particles suspension (0.22-mm filtered) maintained in SM buffer (10^8 PFU/mL phages) on a Formvar-carbon coated copper grid and allow the phages to adsorb for 2 min.
2. After 30–60 s of drying, blot off the excess liquid, and stain the phages by adding drops of 2% PTA solution (pH 7.4), on top of the phage drops.
3. Allow the grid to air-dry for 10 min.
4. Observe the phage morphology under a transmission electron microscope (TEM; JEM-2010; JEOL, Tokyo, Japan) at 200 kV and identify it by comparing the morphology with existing phage morphology data.

3.3.2 By Sequencing of Bacteriophage Genome

Isolate the genomic DNA from selected bacteriophage using the method described by Sambrook and Russell [9] and Pickard [11]

Isolation of Genomic DNA

1. Take concentrated phage suspensions obtained from the 0.22-mm filtered lysates.
2. Treat the lysate with DNase and RNase at the final concentration of 1 mg/mL.
3. Add polyethylene glycol (PEG) 8000 1:10 (w/v) for precipitation of viral particles.
4. Treat the above suspension with 20% SDS and proteinase K (20 mg/mL) to lyse the particles.
5. Give successive treatment of phenol, chloroform, and isoamyl alcohol at 25:24:1 (v/v).
6. Centrifuge it at $7168 \times g$ for 15 min and collect the supernatant containing the genomic DNA.
7. Precipitate the genomic DNA by adding ethanol, progressively at 100% and 70%.
8. Centrifuge it at $11,200 \times g$ for 15 min, discard the supernatant, and air-dry the DNA pellet.
9. Resuspend the DNA pellet in nuclease-free water (NFW) prior to checking DNA concentrations and purities by UV spectrophotometry (260/280 ratio).

Sequencing of Bacteriophage Genome

Sequencing can be carried out by using any high-throughput DNA sequencing platform, such as Illumina SBS Technology [4].

1. Synthesize paired-end libraries with approximate average insert lengths of 200 base pairs from the DNA using the Nextera XT (Illumina) protocol, and assay the library concentrations and sizes on a 2100 Bioanalyzer.

2. Sequence the libraries using an Illumina sequencer HiSeq 2500/1500 (2×150 bp).
3. Analyze the obtained sequences and annotate preliminary after cleaning of the sequences of the FASTQ file.
4. Remove any low-quality sequences (reads with a base quality less than 20) and assemble only the high-quality reads to construct unique consensus sequences by analysis against a reference sequence and de novo genome assembly.
5. Generate a single large contig by reads assembly for each bacteriophage genome. [Note: define genomes as complete if the single contig assembled without error].
6. Use these contigs to identify any closely related homologs with the NCBI BlastN tool by comparison with the sequences published in the GenBank.
7. Perform the preliminary functional annotations of the assembled genomes by sequence comparison with the NCBI public database, and select the best aligning results to annotate the unigenes, if any.

3.4 Screening of Isolated Phages for Biocontrol Activity

3.4.1 Biocontrol of Phages in Environmental Water

In the Absence of Water Microbiota [4]

1. Inoculate *R. solanacearum* strain IVIA-1602.1 (5×10^6 CFU/mL) in 100 mL of 0.22- μ m filtered and autoclaved river water samples.
2. Further, inoculate each of the bacteriophages tested at about 10^4 PFU/mL [multiplicity of infection (MOI) = 0.01].
3. Incubate it at 24 °C for 30 h and 14 °C for 60 h with shaking (200 rpm).
4. Take aliquots for bacterial and phage counts regularly. Carry out serial tenfold dilution in PBS and plate onto YPGA medium.
5. Carry out these assays at least in duplicate and in separate experiments.
6. Record the results.

In the Presence of Water Microbiota [4]

To evaluate whether the appearance of *R. solanacearum* cells resistant to the action of the lytic phages could really take place in the environment, biocontrol assays can be performed in nonsterile environmental water with the whole indigenous microbiota, and monitored in the long term at 24 °C and 14 °C.

1. Process the river water samples with the whole microbiota (protozoa, bacteria, virus, etc.) by enrichment assays for detection of *R. solanacearum* lytic phages that could be naturally present in the water samples.
2. If no lytic activity is observed then, inoculate the water samples with a selected bacteriophage at about 10^4 PFU/mL.

3. Process the volumes of 100 mL in three different ways as follows:
 - (a) 0.22-mm filtration and autoclaving, with no alive microbiota (AW),
 - (b) 0.22-mm filtration, only containing the indigenous phages without activity against *R. solanacearum* and the inoculated phage (FW), or
 - (c) without treatment, with the whole native microbiota and the inoculated phage (W).
4. Inoculate all of the above tubes subsequently with *R. solanacearum* strain IVIA-1602.1 at about 5×10^6 CFU/mL (MOI = 0.01).
5. Incubate it in static conditions at 24 and 14 °C for a month.
6. Take aliquots for *R. solanacearum* counts at inoculation time (day 0), for 30 h at 24 °C and 60 h at 14 °C, and then weekly for a month.
7. Carry out serial tenfold dilution in PBS and plate it onto YPGA medium as mentioned above.
8. Carry out these assays at least in duplicate and in separate experiments.
9. Record the results.

3.4.2 Biocontrol in Host Plants by Watering

With One Single Phage

The ability of a selected river water bacteriophage for bacterial wilt biocontrol in planta can be tested in a susceptible host [4].

1. Take sets of 10 tomato plants or plants of interest (aged 3 weeks).
2. Co-inoculate them in duplicate in two independent experiments (Experiment 1 and Experiment 2) by watering once with 20 mL of *R. solanacearum* strain IVIA-1602.1 (10^4 or 10^5 CFU/mL), and a selected bacteriophage (10^9 or 10^6 PFU/mL) and their tenfold dilutions.
3. Further, perform Experiment 3 in triplicate by watering once with 20 mL of *R. solanacearum* strain IVIA-1602.1 (10^5 CFU/mL) and the same selected bacteriophage (10^8 PFU/mL).
4. Tomato plants inoculated either with the bacterial strain or nonsterile water serve as controls.
5. Maintain all plants in a climatic chamber of adequate dimensions, in cycles of 16 h light 8 h darkness at 26 °C and 70% humidity in optimal conditions for disease development, under Biosafety Level 3 (BSL3).
6. After inoculation, monitor plants periodically for symptom development by visual inspection during 6–8 weeks.
7. Record any wilting symptoms in less than 25% of leaves as first symptoms to be confirmed in subsequent evaluations.

8. Record the progressive increase in wilting incidence to 50–75% of the leaves and branches as positive wilting in the plant.
9. Record more than 75% of leaves and branches affected as near collapse or collapse of the wilted plant.
10. Perform bacterial re-isolation from wilted plants according to protocol described by European Directives [12] in a modified semi-selective medium South Africa (SMSA) agar [13] followed by PCR identification of the colonies [12].
11. Record the results.

With Single Phages and their Combinations

Further, the abilities of selected river water phages for bacterial wilt biocontrol in planta can be tested either alone or taking part of a phage mixture, in a susceptible host in biocontrol assays [4].

1. Take sets of around 35 tomato plants or plants of interest (aged 4–5 weeks).
2. Co-inoculate them in two independent experiments by watering once with 20 mL of *R. solanacearum* strain IVIA-1602.1 (10^5 or 10^6 CFU/mL) and the selected bacteriophages and their combinations (10^7 PFU/mL) (MOIs = 100 and 10, respectively) in all the experimental conditions.
3. Tomato plants inoculated either with the bacterial strain or nonsterile water serve as controls.
4. Incubate the plants (more than 300 per assay) under BSL3 conditions, and monitor for a period of 1.5–2 months.
5. Do the sampling and process it as mentioned above.
6. Record the results as described above.

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Chapter 21

Isolation of Bacterivorous Protozoan, *Acanthamoeba* Spp., as New-Age Agro Bio-Input

Chandrashekhar D. Patil, Bhavana V. Mohite, and Satish V. Patil

Abstract

Protozoan is widely distributed in the environment. *Acanthamoeba* genus are free-living protozoan of nonpathogenic and pathogenic nature. Its intrinsic ability to feed on soil bacteria contributes greatly to the soil nutrient turnover and thereby plant growth. This protocol provides a firsthand guide to isolate *Acanthamoeba* from soil samples and their preliminary identification through staining procedure. The isolates could be further tested for plant beneficial roles in laboratory settings.

Key words Protozoan, *Acanthamoeba*, Agriculture, Cyst, Soil fertility

1 Introduction

Protozoa are single-celled eukaryotic microorganisms. They are heterotrophic and mostly predate on the soil bacteria. They are widely present in water and soil habitat. Through their feeding behavior, they play an important role in recycling of soil organic nutrients. Members of *Acanthamoeba* genus are free-living Protozoa and most predominant bacterivores species in the environment. The classification scheme of *acanthamoeba* genus is as follows:

Kingdom: *Protista*.

Subkingdom: *Protozoa*.

Phylum: *Sarcomastigophora*.

Subphylum: *Sarcodina*.

Superclass: *Rhizopoda*.

Class: *Lobosea*.

Subclass: *Gymnamoebia*.

Chandrashekhar D. Patil and Satish V. Patil contributed equally to this work.

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Order: *Amoebida*.

Family: *Acanthamoebidae*.

Genus: *Acanthamoeba*.

Free-living *Amoebae* of *Acanthamoeba* genus include non-pathogenic and pathogenic strains that are currently classified in 18 different genotypes, T1–T18 [1]. The life cycle of *Acanthamoeba* consists of two stages, metabolically active, trophozoite, and metabolically inactive cyst. Both trophozoite and cysts are characterized by a nucleus surrounded by a dense central nucleolus [2]. The interchange between two life stages depends on the external stimuli such as nutrient depletion or availability. A gram of soil generally contains 10^3 – 10^7 amoeba with varying size $<10\ \mu\text{m}$. The differences between cyst size and shape were the early markers for the differentiation between *Acanthamoeba* spp.

However, distinction between closely similar species was a major challenge due to the irregularity and variation in shapes. Latest molecular tools are highly recommended to profile the soil isolates and characterize their genotypes [3]. Due to the most abundant nature of *Acanthamoeba* in the environment, it is a suitable model organism for soil protozoan function studies [4].

Top organic soil layer (10–15 cm depth) harbors the high number of protozoa and microbial communities. *Acanthamoeba* voraciously feeds on soil bacteria at a rate from 0.2 to 1465 bacterial h^{-1} [5, 6]. Therefore, presence of protozoan, microorganisms, and soil nutrition quality are closely associated with conditions for the successful nutrient turnover via formation of “microbial loop in soil” [7]. Plant root exudates contain the nutrient that triggers the bacterial growth through assimilation of carbon and nitrogen in the rhizosphere. The abundance of bacteria in rhizosphere favors the progression of bacterivorous protozoan and ultimately regulation of bacterial population in soil. Protozoa preying on bacteria thus make nitrogen available for plant growth [4].

Considering the potential of bacterivorous protozoan in organic farming and as a next generation of agriculture bio-inputs [8], this chapter outlines the practical steps to isolate the *acanthamoeba* spp. as a model organism and their preliminary identification.

2 Material and Methods

2.1 Requirements

1. Soil sample.
2. Minimal nutrient medium (peptone: 1.25 g/l, yeast extract: 1.25 g/l, dextrose: 3.0 g/l, agar: 20.0 g/l).
3. Petri plates.
4. *E. coli* culture.

5. Autoclave.
6. Heating chamber.
7. Microscope.
8. Staining reagents.

2.2 Culture Medium Preparation

Acanthamoeba spp. can be easily cultivated on non-nutrient agar or agar media containing minimal concentrations of nutrients. Sterilize the medium by autoclaving at 15 lb. pressure for 20 min, allow the medium to cool at 45 °C, then pour the medium into petri plates, and allow them to solidify for 1 h at room temperature or 30 °C for 2 h. After solidification, aseptically overlay 1 ml of 24 h old culture of *E. coli* heated at 60 °C for 10 min, then incubate the plates at room temperature for 1 h.

2.3 Collection and Plating of Soil Sample

In sterile plastic bags, collect 1 g of rhizospheric soil from the irrigated soil/farms and crop plants as per need. Close the bag and label the sample appropriately (e.g., location, crop, date, etc.). The sample should be store at 4 °C until further experimentation (*see* **Note 1**).

2.4 Primary Identifications

The cultivated bacterivorous protozoan can be identified by observing loopful of culture with loopful of water under 40×, note down the structure (Fig. 1). The *Acanthamoeba* cyst and trophozoite stage can be identified by using simple staining methods like methylene blue, cotton blue, and Giemsa staining.

2.5 Detail Procedure

2.5.1 Part I

1. Dilute the collected rhizospheric soil with sterile saline by routine serial dilution techniques up to 10⁹ dilution and mix well.
2. Aseptically remove 0.5 ml from diluted sample up to 10⁶ and plate it on sterile culture medium, and spread the sample by rotating plates clockwise and anticlockwise to spread sample uniformly.
3. Incubate plates at 30 °C for 24–72 h.
4. At every 24 h, take loopful of culture and dilute with drop of sterile water.
5. Put this drop carefully at the center of cavity slide with wax at four corners, and invert the slide to make hanging drop preparation and observe under microscope at 40×.
6. Note down the shape and structure of cultivated protozoan (*see* **Note 2**).
7. Further observation for identification can also be done by using methylene blue, cotton blue, iodine, and Giemsa staining (*see* **Notes 3–7**).

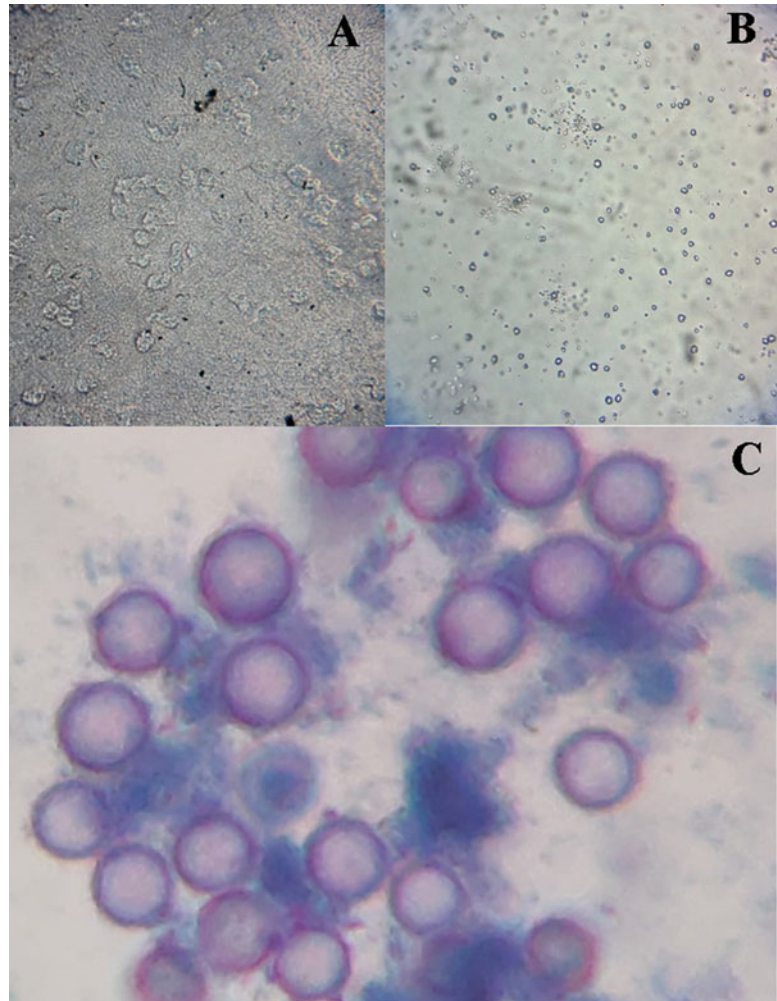


Fig. 1 Microscopic observation of different *Acanthamoeba* life forms. (a) *Acanthamoeba* cyst (10 $\times$). (b) Free-living trophozoites of *Acanthamoeba*. (c) Stained *Acanthamoeba* cyst (methylene blue staining, 40 $\times$)

2.5.2 Part II: Staining

1. Take a loopful culture of protozoan and spread on a glass slide and gently heat on the flame.
2. Flood the fixed smears of protozoan cyst with freshly filtered carbol fuchsin solution.
3. Then wash the smear in tap water and flood the smear with malachite green as counterstain for 10 s.
4. Then rinse the smear again in tap water and air-dry.

2.5.3 Part III

Axenic Culture of
Acanthamoeba

The pure culture of *Acanthamoeba* can be done by following steps:

1. Harvest the cysts from the peptone–yeast extract–dextrose (PYD) plate cultures by adding 5 ml sterile saline in 2-week-old *Acanthamoeba* seeded plates (*see Note 8*).
2. Collect the suspension and then centrifuge at 7000 rpm (centrifugal g force 16052). For 10 min, then collect the pellet.
3. Incubate the pellet with 3% HCl overnight for elimination of the bacteria.
4. Then wash the cysts 2–3 with physiological saline (7000 rpm for 10 min) to remove remaining HCl.
5. Then transfer it to liquid cultures sterile PYD medium.
6. The culture should be maintained axenic by transferring every second week to fresh sterile PYD medium.

3 Notes

1. Since *Acanthamoeba* is both pathogenic and nonpathogenic in nature, appropriate safety measure should be taken to handle the samples. Sample collection from natural sites with minimum human exposure should be preferred for the isolation of nonpathogenic *Acanthamoeba* spp.
2. Cysts can be counted using a hemocytometer.
3. Basic fuchsin stain in aqueous solution with phenol and ethanol will stain *Acanthamoeba* cysts in red/magenta color against a greenish-blue background.
4. Eosin, iodine, and methylene blue stains are useful for wet mount staining. Eosin stain can be prepared by adding 1 g eosin in 100 ml distilled water, and 0.5 ml acetic acid.
5. Prepare methylene blue stain by dissolving 1 g of methylene blue powder in 100 ml physiological saline.
6. For wet mount staining, spread two drops (25–30 μ l) of *Acanthamoeba* suspension on a glass slide; add a drop of the stain, cover with glass coverslip, and observe under microscope at 40 $\times$ power.
7. The all above staining methods allow to observe cysts of *Acanthamoeba* known to form double-walled cysts, the outer wall ectocyst which is differentiated from the variably stained surrounding background. The inner wall appears as stellated, polygonal, round, or oval forms.
8. Sodium dodecyl sulfate (SDS, 0.5% final concentration) can be used to confirm the transformation of trophozoites into cysts. Trophozoites are SDS-sensitive and any remaining are lysed immediately upon addition of SDS, while cysts remain intact (Dudley, 2005).

Acknowledgments

We acknowledge Prof. Michael Bonkowski, University of Cologne, Germany for pioneering the work in ecosystem engineers in shaping rhizosphere microbial diversity and functioning and their feedback on plant productivity. We are also thankful to our research collaborator Prof. Marilise B Rott, Universidade Federal do Rio Grande do Sul, Brazil for inspiring us on protozoan study.

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Part II

Isolation of Mineral Solubilizing Microbes



Isolation and Screening of Zinc Solubilizing Microbes: As Essential Micronutrient Bio-Inputs for Crops

Satish V. Patil, Hemant P. Borase, Jitendra D. Salunkhe, and Rahul K. Suryawanshi

Abstract

Zinc is indispensable micronutrient for optimum plant growth. Soil is a rich source of zinc but major portion of it is in the form of insoluble complexes and therefore is inaccessible to plants. This inorganic zinc can be converted to soluble form (divalent cation) with the help of zinc solubilizing microbes. Microbes solubilize zinc through different mechanisms including acidification, oxidoreductive reactions, or production of siderophores. Based on zinc solubilization property, microbes can be isolated from soil by the use of selective minimal media containing zinc. The zone of clearance formed because of zinc solubilization can confirm the presence of zinc solubilizing microbe. Finally, the quantification of solubilized zinc can be made by atomic absorption spectroscopy.

Key words Micronutrient, Oxidoreductase, Clinical biomarker, Solubilization

1 Introduction

Micronutrients are essential components required for the optimum growth of living organisms. Among diverse micronutrients, zinc (Zn) is abundant in the biosphere and performs an indispensable role in the metalloenzymes activity [1, 2]. Zinc is a unique element that takes part in all types of enzymatic catalysis reactions such as oxidoreductase, transferase, hydrolase, lyase, isomerase, and ligase [3]. Distinguished chemical properties of zinc-like speedy ligand exchange, Lewis acidity, and others allow its selection over other metals as structural components in several (around 300) biological proteins including transcription factors having zinc fingers, superoxide dismutase (SOD), and carbonic anhydrase (CA) [4].

In different metabolic and physiological processes (synthesis of starch and protein, formation of indole-3-acetic acid (IAA)), zinc

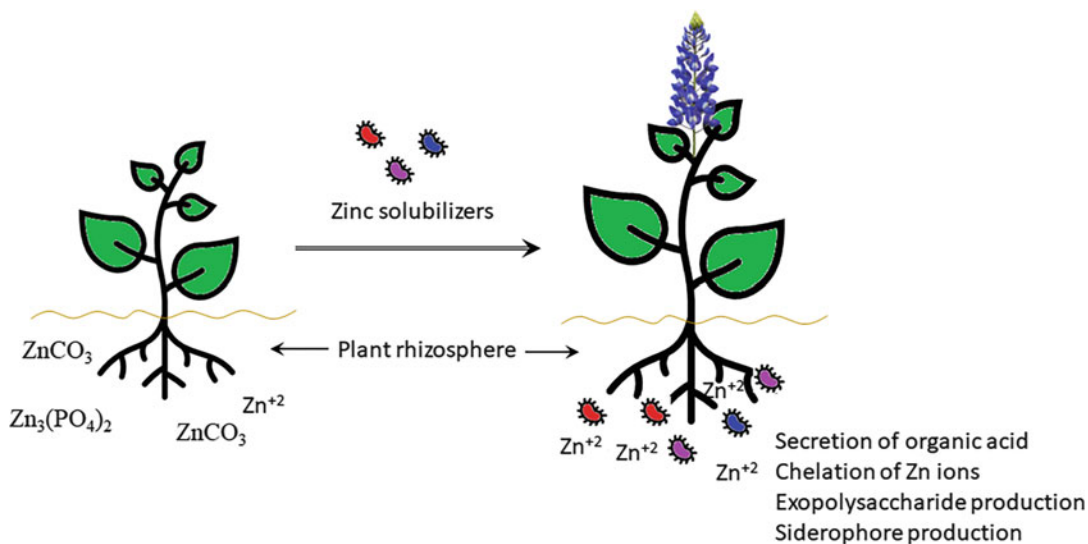


Fig. 1 Graphical representation of zinc solubilizing bacteria for improving zinc bioavailability through direct and indirect mechanism of action

plays the foremost role [5, 6]. Zinc is also emerging as new target against bacterial pathogens and as a clinical biomarker [7, 8].

Bacterial cell tightly regulates the intracellular optimum zinc concentration to avoid metal toxicity, as a marginal increase in zinc inhibits metabolism and vital cellular functions [9]. For this purpose, some of the metal regulation strategies are adopted by bacteria which involve metal uptake with high and low affinity, efficient export system to remove excess metal, and safe storage of lightly bound metal for future use [10]. Similarly, the role of zinc binding and transporting proteins is very important for normal zinc metabolism [9]. The exact mechanism of zinc solubilization by bacteria is not clearly revealed, but cell wall modification, efflux, precipitation, and synthesis of organic acids are some of the proposed ways for zinc utilization [11]. Synthesis of organic acids is claimed to have remarkable effects on metal solubilization via release of other metabolites [12]. Organic acids such as citric, lactic, formic, oxalic, and glycolic acid are known for chelating properties and reported for solubilizing certain metals [13] (Fig. 1).

We can make use of these zinc solubilization properties of microbes to screen them from their natural resources. Here we represent a protocol for screening of zinc solubilizing microbes from plant rhizosphere.

2 Principle of Assay

Plant rhizosphere is a rich source of zinc solubilizing microorganisms. Screening of zinc solubilizers from plant rhizosphere uses a classical approach of cultivating test microorganisms on zinc oxide

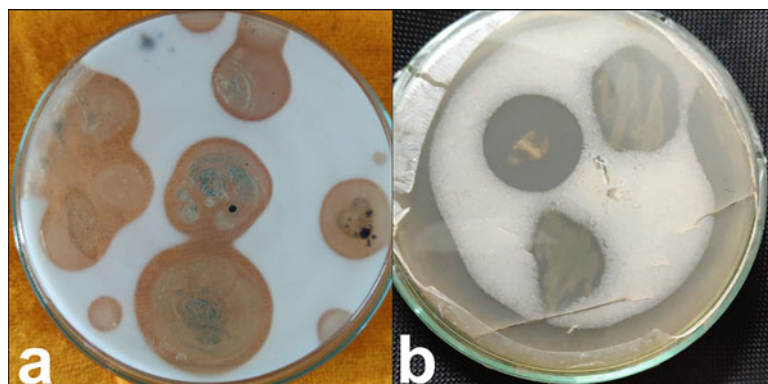


Fig. 2 The potent zinc solubilizing fungi (a) and bacteria (b) (From Dr. SVP, Biochemistry, KBCNMU, Laboratory)

containing minimal medium and isolating microbes producing zone of clearance (Fig. 2). The diameter of zone of clearance is relative to its capacity of zinc solubilization. The solubilized zinc can further be estimated through atomic absorption spectroscopy.

3 Materials

1. Rhizospheric soil/root nodules, etc.
2. Sterile containers for soil sample collection.
3. Sterile tubes for serial dilution of soil samples.
4. Zinc solubilizing isolation medium:
 - (a) Basal medium containing (g/L): Glucose, 10.0; $(\text{NH}_4)_2\text{SO}_4$, 1.0; KCl, 0.2; K_2HPO_4 , 0.1; MgSO_4 , 0.2; double distilled water, 1000 mL; and buffered to pH 7.0.
 - (b) General insoluble source of zinc: Zinc oxide. Use 0.5% concentration, sterilize separately and then add in the sterile basal medium. Besides this, other zinc salts such as $\text{Zn}_3(\text{PO}_4)_2$ and ZnCO_3 can also be used as substrate at 0.2% concentration.
5. Sterile nutrient agar and/or potato dextrose agar.

4 Method

4.1 Collection of Soil Samples

1. Collect the soil samples/root nodules from plant rhizosphere at different locations.
2. Carry the soil samples to the lab in cold and sterile container and store the samples at 4 °C until further use.
3. Bring soil to the room temperature and measure about 1.0 g of soil sample and mix with 9 mL sterile saline, make up the

volume to 10 mL. Serially dilute the resultant suspension till 10^9 dilutions.

4. For root nodules, surface-sterilize them with 0.01% HgCl_2 or 70% ethanol. Select pink nodules, cut and crush in sterile saline followed by serial dilution using sterile saline as per previous step.

4.2 Screening of Zinc Solubilizing Microorganisms

1. Prepare petri plates containing sterile modified minimal agar medium fortified with complex zinc.
2. When the petri plates are ready, take a loopful suspension from 10^4 to 10^9 dilution of soil or nodule and streak it the Petri plates. Incubate the plate at 28°C till 24–96 h. Keep observing the plates for the zone of zinc solubilization around the microbial colony.
3. Select the microbial colony(ies) causing zinc solubilization and purify it (them) on nutrient agar or on potato dextrose agar (PDA) plates.
4. Inoculate pure culture spot of an organism at the center of sterilize zinc solubilizing agar plate and incubate up to 24 h to 1 week, and measure zone of solubilization as a function of time (Fig. 2).
5. Make pure culture of potent organism. Bacterial cultures can be identified by using Gram staining, biochemical test (Bergey's Manual of Systematic Bacteriology), and molecular identification (16S rRNA), while fungal isolates can be identified by microscopic observation of hyphal and spores morphology and molecular identification (18S rRNA, FAME, etc.).

4.3 Qualitative Estimation of Zinc Solubilizing Potential by Liquid Culture Method

The zinc solubilizing microbial isolates showing clear zone formation (Fig. 2) can be further tested for their ability to release inorganic Zn in a liquid medium. For the quantitative assay, transfer 20.0 mL of liquid mineral salts containing (g/L) dextrose, 10.0; $(\text{NH}_4)_2\text{SO}_4$, 1.0; KCl, 0.2; K_2HPO_4 , 0.1; MgSO_4 , 0.2; pH 7.0 and insoluble Zn compounds ($\text{Zn}_3(\text{PO}_4)_2$, ZnO, and ZnCO_3 : 100 mg/L each, in 50 mL Erlenmeyer flask. After autoclaved the medium at 121.0°C for 20 min, transfer 1.0 mL suspension of individual pre-identified bacterial culture to the flask and incubate at shaking condition at 30°C and 120 rpm. For each isolate, prepare four replications along with an uninoculated control. After 24 h of incubation, aseptically transfer the mixture to centrifuge tubes and centrifuge at $10000 \times g$ for 15 min at 4°C . Collect the supernatant in a test tube and transfer 1.0 mL of the supernatant to 50.0 mL volumetric flask. Make up the volume to 50.0 mL using distilled water. Estimate soluble Zn content in sample using atomic absorption spectroscopy (AAS)/Fourier transform infra-red (FTIR) techniques.

The procedure can be further repeated for more time intervals (second day, third day, fifth day, and tenth day).

4.4 Qualitative Estimation of Zinc Solubilizing Potential by Solid Plate Method

Prepare sterile zinc agar plates and inoculate the loopful of pure culture suspension as spot at center of plate. Leave the plate as it is for 20 min and then move it to incubator for 48 h for bacterial culture and 3–4 days for fungi. After incubation, measure the diameter of microbial growth and zone of clearance. Measure the diameter of the clear zone and colony growth diameter. Determine zinc solubilization efficiency by following formula:

$$\text{Solubilization efficacy} = \frac{\text{Solubilization diameter}}{\text{Diameter of colony growth}} \times 100$$

5 Notes

1. Any insoluble form of zinc can be used in zinc solubilization agar medium, i.e., zinc phosphate ($\text{Zn}_3(\text{PO}_4)_2$), zinc oxide (ZnO), and zinc carbonate (ZnCO_3) at minimum concentration 100 to 0.5 mg/L. [14].
2. In case of solid agar medium, separately sterilize the insoluble zinc sources and after sterilization add into basal medium with proper mixing.
3. ZnO more effectively solubilize than other zinc complexes, i.e., ZnCO_3 .
4. Zinc solubilization occurs due to increase in H^+ concentration, i.e., acid production and also by ammonia assimilation [14].
5. The pH-dependent mechanism of zinc solubilization for a particular microbe, may be determined using acidic or alkaline pH indicator in the medium.

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Isolation and Screening of Mineral Phosphate Solubilizing Microorganisms

Swati Patel, Vimalkumar Prajapati, and Pritesh Patel

Abstract

Phosphorus is abundant in soil but its availability is limited due to its immobilization and complexation with mineral ions. The sources of phosphate are sustainable only when they are in receipt of solubilized by rhizospheric microorganisms. Phosphate solubilizing microorganisms (PSMs) such as rhizospheric or endophytic bacteria, free-living fungi, or vesicular-arbuscular mycorrhiza (VAM) have potency to solubilize mineral phosphate source by secretion of acids, enzymes, chelation of metal ions, and H^+ ion extrusion. The isolation and screening of these PSMs is done on Pikovskaya medium, NBRIP medium, or Tris minimal agar medium with application of different sources of phosphate like tricalcium phosphate, apatite, or rock phosphate and detected by either zone of solubilization or color of zone around the colony. The amount of released phosphate in the solution is determined by measuring the reduction of phosphomolybdate or vanadomolybdate complex in acidic condition. The amount of reduced complex is directly proportional to soluble phosphate.

Key words Mineral phosphate solubilization, Phosphate solubilizing microorganisms, Organic acid, Chelation, Phosphomolybdate complex

1 Introduction

Phosphorus is key requirement of plant after nitrogen for the metabolic activities like photosynthesis, energy transfer, catabolism, and anabolism of carbohydrate. Plant can uptake P in form of $H_2PO_4^-$ or HPO_4^{2-} but greater part of P in soil is in form of minerals. Phosphate is found to form complexes with different metal ions like Ca, Al, Zn, and Fe depending upon the nature and type of soil [1]. Rhizospheric microorganisms are the candidates who can solubilize this mineral phosphate and make them accessible to plants by various mechanisms like secretion of low molecular weight organic acids, excretion of H^+ ions, synthesis of different enzymes, chelation, and ion exchange. The microorganisms solubilize insoluble phosphate sources like tricalcium phosphate and rock phosphate into solubilized form by secretion of various

organic acids like gluconic acid, ketogluconic acid, oxalic acid, citric acid which chelate cations like Ca^{2+} , Al^{2+} , Fe^{2+} , Fe^{3+} and loosen the cation–oxygen bond of mineral phosphate by the hydroxyl and carboxyl groups or via secretion of H^+ ions [2, 3]. These organic acids are also exported out of the cell by H^+ symport transport assembly which reduces the pH of outer environment. Excretion of H^+ by microorganisms into rhizosphere is also caused by higher uptake of cations such as nitrogen (NH_4^+) and leads to reduction in the pH in its vicinity to solubilize phosphate [4].

In molybdenum blue method, the phosphate present in the solution reacts with ammonium molybdate and forms a phosphomolybdate complex. Ascorbic acid is a reductant and reduces this complex which is directly proportional to the amount of soluble phosphate present in the solution. In vanadomolybdate yellow method, under acidic condition orthophosphate reacts with ammonium molybdate and ammonium metavanadate and forms a yellow-colored ammonium phosphoric vanadomolybdate complex which is detected spectrophotometrically.

The phosphate solubilizing bacteria (PSB) are enlisted as species of *Pseudomonas*, *Enterobacter*, *Serratia*, *Bacillus*, *Burkholderia*, *Pantoea*, *Rhizobium*, *Sphingomonas*, *Acinetobacter*, and *Achromobacter* [2]. Phosphate solubilizing fungi (PSF) are found to be more superior in terms of root colonization and phosphate solubilization, and the prominent genus are *Penicillium*, *Aspergillus*, and *Trichoderma*.

2 Materials

2.1 Isolation and Screening of Phosphate Solubilizing Microorganisms

1. Pikovskaya medium (pH 7.0): Glucose (carbon source) 10.0 g/L, $(\text{NH}_4)_2\text{SO}_4$ 0.5 g/L, KCl 0.2 g/L, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 1.0 g/L, MnSO_4 0.0001 g/L, FeSO_4 0.0001 g/L, yeast extract 0.5 g/L, and tricalcium phosphate 5.0 g/L [5].
2. NBRIP (National Botanical Research Institute Phosphorus) medium (pH 7.0): Glucose 10 g/L, $(\text{NH}_4)_2\text{SO}_4$ 0.1 g/L, KCl 0.1 g/L, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.25 g/L, $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ 0.25 g/L, and $\text{Ca}_3(\text{PO}_4)_2$ 5.0 g/L or hydroxyapatite 5.0 g/L [6].
3. 100 mM Tris–Cl (pH 8.0) buffered minimal medium with 100 mM glucose as sole C source with MgSO_4 2 μM , NH_4Cl 10 mM, and micronutrients: $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ 3.5 mg/L, $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ 0.16 mg/L, $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ 0.08 mg/L, H_3BO_3 0.5 mg/L, $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ 0.03 mg/L, $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ 0.4 mg/L, rock phosphate 1 mg/mL [7]. Methyl red is added into the media in concentration of 0.03% to observe color change.

2.2 Quantitative Estimation of Phosphate by Ascorbic Acid Method

1. Solution I: Ascorbic acid 10% (store the solution at 4°C for a month).
2. Solution II: Ammonium molybdate ((NH₄)₆. Mo₇O₂₄. 4H₂O) 0.42% in 1 N H₂SO₄ (take 28.6 mL of conc. H₂SO₄ with 4.2 g of ammonium molybdate and make up 1000 mL with distilled water) (store the solution at room temperature).
3. Solution III: Mix 1 part of Solution I to 6 parts of Solution II and store in ice bath for assay.

2.3 Quantitative Estimation of Phosphate by Vanadomolybdate Method

1. Solution I: Ammonium molybdate ((NH₄)₆. Mo₇O₂₄. 4H₂O) 10.0 g dissolve it in 200 mL of warm distilled water.
2. Solution II: Ammonium metavanadate ((NH₄)₂ VO₃) 0.5 g dissolve it in 200 mL of warm distilled water.
3. Solution III: Mix Solution I and Solution II and add 125 mL of concentrated HNO₃ (solution can be stored at 4 °C for an indefinite period).

3 Methods

3.1 Isolation and Screening of Phosphate Solubilizing Microorganisms

1. Collect rhizospheric soil samples from the nonfertilized area. Discard the soil limply adhered to roots and suspend 1 g of soil attached to the roots into 10 mL saline.
2. Enrichment: Mix 1 g of soil sample with 10 mL of saline solution and keep it under rotatory shaker for 1 h in sealed flasks. This soil suspension is left uninterrupted. Allow it to settle appropriately before more processing.
3. Take 1 mL of supernatant and go for serial dilution up to tenfold [8] and spread it on either of the screening media. Incubate the plates for 24 h.
4. The phosphate solubilizing isolates show clear zone on Pikovskaya medium (Fig. 1a, b), and NBRIP medium (Fig. 1c, d). The Tris minimal media supplemented with methyl red shows pink colored zone around the colony (Fig. 1e, f) due to acidification of medium.
5. The solubilization index is determined by using following formula [9].

$$\text{Solubilization index (SI)} = (\text{halo zone} + \text{colony diameter}) / \text{colony diameter}.$$
6. Purify the colonies which show either zone of solubilization or colored zone and process it for quantitative estimation by growing them in respective liquid media with different phosphate source.

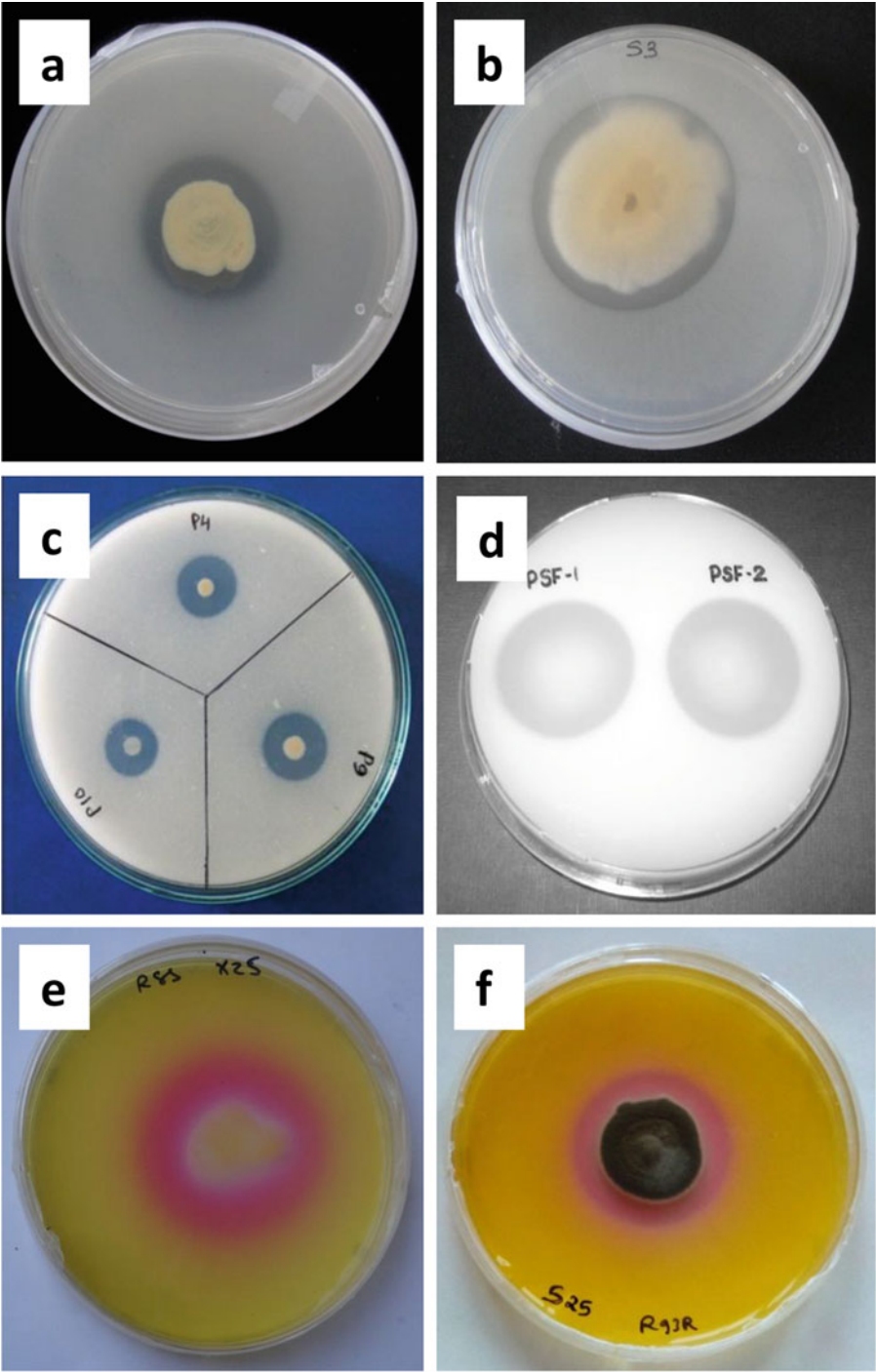


Fig. 1 Solubilization of insoluble phosphate by bacterial and fungal isolates on Pikovskaya medium (**a** and **b**), National Botanical Research Institute's phosphate (NBRIP) growth medium (**c** and **d**) [10, 11], 100 mM Tris-Cl (pH 8.0) buffered minimal medium with 100 mM glucose as sole C source and methyl red (**e** and **f**)

7. Inoculate the flasks with pure colony and incubate it. Check for the pH regularly. Collect the samples from the flask which shows acidic pH and estimate for soluble phosphate.

3.2 Quantitative Estimation of Phosphate by Ascorbic Acid Method [12]

1. Take 0.7 mL of Solution III and add 0.3 mL of test solution in test tube. Use 0.3 mL of water for the blank.
2. Incubate the mixture at 45 °C for 20 min or at 37°C for 1 h and observe for color development.
3. Measure optical density at 820 nm (0.01 micromole of phosphate gives optical density of 0.260 at 820 nm).
4. Use a solution of KH_2PO_4 as a standard.

3.3 Quantitative Estimation of Phosphate by Vanadomolybdate Method [13]

1. Take 1 mL of test solution into test tube.
2. Add 4 mL of Solution III into solution and check for the development of yellow color (*see Note 1*) and record optical density at 420 nm against blank (*see Notes 1–5*).
3. Use a solution of KH_2PO_4 as a standard.

4 Notes

1. The color developed during assay is stable for several hours.
2. The optical density is proportional to phosphate concentration up to factor 1.8.
3. As this method is sensitive, make all the glassware used in experiment free of phosphate traces.
4. Use a separate cuvette for phosphate estimation.
5. This method can measure the concentration of 0.01 micromole of phosphate.

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Chapter 24

Isolation and Identification of Potassium-Solubilizing Microbes

Prittesh Patel and Swati Patel

Abstract

Potassium-solubilizing microorganisms (KSM) could serve as inoculants that convert insoluble potassium in the soil into a form that plants can get to. Lack of K generally brings about a decline in harvest yields. Utilizing K-solubilizing microorganisms to expand the concentration of accessible K particles in the soil may relieve K inadequacy. Potassium-solubilizing microbes develop on Aleksandrov medium and form a clear zone around the state, shaped because of potassium solubilization in the region of the settlement. By breaking down silicate minerals, for example, K-feldspar and mica, KSB change strong K in the dirt into accessible K that can be straightforwardly consumed by plants, and they secrete dynamic substances that promote plant development.

Key words Potassium-solubilizing microbes, Aleksandrov medium, Mica powder, Photosynthesis, Plant

1 Introduction

Potassium (K) is basic component for plant growth promotion. It is concerned about the change of plant cell osmotic pressure and the transportation of compounds in plants. It promotes the activation of enzymes, the usage of nitrogen (N), and the amalgamations of protein and sugar. It likewise helps plant photosynthesis process in plants [1]. In the soil, K exists fundamentally in four unique pools, that is, mineral, non-replaceable, interchangeable, and dissolvable K. In plants, K insufficiency causes yellowing of the leaf edges, giving them a burned appearance. It can likewise cause moderate development and deficient root improvement [2]. The capacity of a couple of soil microscopic organisms to change inaccessible types of potassium to an accessible structure is a significant element in plant

growth promoting microorganisms for expanding crop yields of high-K-demand crops. Soil microorganisms can deliver soluble K from K-bearing minerals, for example, K-feldspar, mica, illite, and orthoclase. This solubilization could be credited to discharging natural acids which either straightforwardly dissolves rock K or chelate silicon particles to bring K into solution. A wide scope of microbes to be specific *Pseudomonas*, *Burkholderia*, *Acidithiobacillus ferrooxidans*, *Bacillus mucilaginosus*, *Bacillus edaphicus*, *B. circulans*, and *Paenibacillus* sp. has been accounted for potassium release in available form from potassium bearing minerals in soils.

2 Materials

1. Sources of K can be feldspar, mica, illite, potassium aluminum silicate, silicate minerals (muscovite and biotite) to supplement in various growth media in 0.2–1% range.
2. Aleksandrov medium: 0.5% glucose, 0.05% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.0006% FeCl_3 , 0.06% CaCO_3 , 0.2% CaPO_4 , 3% agar pH 7.0 and phenol red dye as indicator [3].
Add K Source in 0.2–1% range.
3. Solid nutrient agar: 0.3% beef extract, 0.5% peptone, 18% agar, pH 7.0–7.5.

3 Methods

1. Collect the soil sample aseptically in sterile Ziploc bags to minimize any possible exogenous microbial contamination. Use immediate or stored at -20°C for future analysis.
2. Take 1 g soil sample and place in the 10 ml sterile distilled water. Shake for 30 min and allow standing for 30 min so that large soil particles get settle down. Use supernatant for the isolation of microbes.
3. Serial dilute up to sixfold to tenfold and inoculate/spread on Aleksandrov agar medium/nutrient agar medium supplemented with K source and incubate the plates at $28 \pm 20^\circ\text{C}$ till the appearance of colonies.
4. Select the isolates on the basis of zone formed around the colonies.

4 Notes

1. Enrichment: Mix the collected soil sample with mica powder and keep at room temperature for 1 week. Withdraw 1gm of soil sample and inoculate in 90 ml liquid media containing 0.95% glucose, 0.045% yeast extract and 0.45% Mica or any K-rich substrate. Make final volume to 100 ml and incubate at room temperature at 120 rpm for 7 days [4].
2. Use deionized sterile water to prepare the media.

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Isolation and Identification of Sulfur-Oxidizing Bacteria

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Abstract

Chemolithotrophic sulfur-oxidizing bacteria (SOB) play a key role in the sulfur cycle in natural and anthropogenic environments because of their enormous capacity to transform many sulfur compounds toxic to the environment. Sulfur is an essential element needed by plants and microorganisms. The SOB application leads to enhancement in the rate of natural oxidation of sulfur and sulfate ion production, which is readily available to the plants at any critical stage subsequent to increase plant yields. Although typically enrichment and isolation of SOB from the environment are done using the Most Probable Number (MPN) method for a long, the spread plate technique and direct plating technique have also been found to be effective, as discussed in this protocol chapter. Here we describe the use of different efficient media, and the use of pH indicator dyes in the isolation medium helps in the easy detection of a SOB. The identification of the SOB is mainly done using the 16S rRNA sequencing technique. It comprises the mapping of the sequence to the available databases followed phylogenetic analysis to infer the precise taxonomic assignment of the isolate.

Key words Sulfur-oxidizing bacteria (SOB), Chemolithotrophs, Enrichment, Isolation, PCR, Sulfate, 16S rRNA gene

1 Introduction

The biological sulfur cycle is mainly carried out by microbial activities, of which sulfur-reducing bacteria and sulfur-oxidizing bacteria are two main players. In the global sulfur cycle, biological oxidation of hydrogen sulfide (H_2S) is one of the key reactions driven by sulfur-oxidizing bacteria [1]. In natural neutral environments such as natural water bodies, forest, and paddy field soils, the presence of the H_2S and its reduced ionic forms are rare. It is caused mainly due to lack of dissolved oxygen and biological anaerobic decomposition of organic compounds containing sulfur or by reduction of elemental sulfur, thiosulfates, sulfate, and sulfites by sulfur-reducing bacteria. Whereas in natural or anthropogenic acidic environments such as geothermal vents and mine-impacted areas, elemental sulfur

is profoundly present [2]. The removal of sulfide, elemental sulfur, and other inorganic sulfur containing compounds from the environments is mainly carried by two groups of photoautotrophic (green sulfur-oxidizing bacteria and purple sulfur-oxidizing bacteria) and chemolithotrophic (colorless sulfur-oxidizing bacteria). The majorly investigated and most abundant SOB are categorized in the family of *Thiobacillaceae*, specifically in terrestrial environments, and in the family *Beggiatoaceae* in aquatic environments [3]. The first acidophilic sulfur-oxidizing microorganism *Acidithiobacillus thiooxidans* (syn. *Thiobacillus thiooxidans*) was isolated and reported by Waksman and Joffe [4].

According to the agricultural view aspects, sulfur is considered to be the fourth major plant nutrient after N, P, and K. It acts as the key element for higher pulse production and plays an important role in the formation of proteins, vitamins, and enzymes [5]. Plants usually take up the sulfur in the form of sulfate, and SOB enhances the natural oxidation process of the sulfur which eventually leads to the production of sulfate, vital for increasing plant yield. Chemolithotrophic sulfur-oxidizing bacteria (*Thiobacillus*, *Thiobrix*, *Sulfolobus*, *Beggiatoa*, and *Thermothrix*) are considered more appropriate from the technological viewpoint as they are known to have the highest rate of sulfide-oxidation, very high affinity towards oxygen and sulfides, and also moderate nutritional requirements [6]. In the present protocol chapter, we have described the application of various efficient isolation media and the solicitation of pH indicator dye which helps in the easy detection of sulfur-oxidizing bacteria (SOB). The spectrophotometric method has been employed to assess the sulfate ion production by sulfur-oxidizing bacteria, and SOBs have been identified using the robust molecular technique.

2 Materials

2.1 Sample Collection for the Screening of the Sulfur-Oxidizing Bacteria

Sample collection can be carried out from any ecological niche including rhizosphere soil, sewage water, tannery effluent, water bodies, etc. The samples are collected in sterile polythene bags and transported to the laboratory in icebox.

2.2 The Culture Media for the Isolation of Sulfur-Oxidizing Bacteria Include

1. Starkey broth (g/L): 3.0 g KH_2PO_4 , 0.2 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2 g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.5 g $(\text{NH}_4)_2\text{SO}_4$, traces of FeSO_4 , with pH adjusted to 8.0. 10 g Elemental sulfur is added to Starkey broth after sterilization [7].
2. Thiosulfate broth (g/L): 5.0 g $\text{Na}_2\text{S}_2\text{O}_3$, 0.1 g K_2HPO_4 , 0.2 g NaHCO_3 , 0.1 g NH_4Cl , with pH adjusted to 8.0.

3. Sulfur-oxidizer medium (SOB) (g/L): 10 g Bacto-peptone, 1.5 g K_2HPO_4 , 0.75 g ferric ammonium citrate, 1.0 g $Na_2S_2O_3 \cdot 5H_2O$, 15 g agar pH 7 adjust using 1 M HCl.
4. Thiosulfate agar medium (g/L): 5.0 g $Na_2S_2O_3 \cdot 5H_2O$, 0.1 g K_2HPO_4 , 0.2 g $NaHCO_3$, 0.1 g NH_4Cl , 15 g agar, and 0.0025 g Bromophenol blue, pH 8.

2.3 Other Chemicals and Requirements

1. Bromocresol purple/Bromophenol blue is used as the indicator.
2. 0.85% NaCl (Saline).
3. Spirit.
4. Incubator, Autoclave, Laminar Air flow.
5. Sugar tubes, glass spreader, sterile petri plates, Erlenmeyer flasks.
6. Sterile propylene tube, wireloop.
7. Sterile polythene bags, ice box for storing the samples.

2.4 Identification of SOB Using Sequencing [8]

1. Thiobacillus medium (g/L): 0.4 g $(NH_4)_2SO_4$, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.25 g $CaCl_2$, 4 g KH_2PO_4 , 0.001 g $FeSO_4$, 5 g $Na_2S_2O_3$, 12.5 g agar, 1 L distilled water.
2. Phosphate buffer saline, 1 × EDTA Tris buffer (pH 8.0), phenol:chloroform.
3. Isoamyl alcohol, isopropanol, Milli-Q water.
4. PCR mix (dNTP, $MgCl_2$, Taq polymerase, sterile distilled water, template DNA).
5. Eppendorf tubes, Dry bath, PCR thermal cycler.
6. 16S rRNA forward 27F (5'-AGAGTTTGTATCCTGGCTCAG-3') and reverse 1544R (5'-AGAAAGGAGGTGATCCAGCC-3') primers.

3 Methods

3.1 Isolation of Sulfur-Oxidizing Bacteria (SOB) [9, 10]

1. To carry out the screening of the sulfur-oxidizing bacteria (SOB), prepare Starkey broth, and thiosulfate broth separately and autoclave it.
2. In the case of the Starkey broth, add 10 g of elemental sulfur after medium sterilization.
3. Under aseptic condition, add 1–3 g of soil samples/1–3 mL of water sample (according to the sampling type) to the 50 mL of broth dispense in Erlenmeyer/Conical flasks.
4. Incubate the flasks in BOD incubator at 32 °C for approximately 25 days.

5. For further enrichment and potential screening, the enriched isolates are subjected for purification by transferring to fresh broth twice at fortnightly intervals.
6. Attain the individual, purified colonies by streaking/or spread method on thiosulfate agar medium.
7. Preserve the single colonies on thiosulfate slants.
8. Properly label the pure cultures and use for characterization and further studies.

3.2 Screening of (SOB) Isolates by pH Reduction Test

1. To perform the pH reduction test for the screening of SOB, inoculate the pure cultures in the 50 mL of growth media in Erlenmeyer flasks with initial pH adjusted to 8.0.
2. Incubate the inoculated flasks at room temperature for 25 days.
3. After incubation, measure the final pH of growth media using a pH meter.
4. Screen the isolates for their efficacy to reduce the pH from 8.0 to 5.0 or less than pH 5.0. Preserve the selected isolates for further studies as well as carry out their morphological and biochemical characterization.

3.3 Qualitative Assessment of Sulfur-Oxidizing Bacteria Using Indicator Dye

1. For initial screening, according to the type of samples, i.e., homogenize the soil sample properly in a 10 mL of sterile saline and dilute serially. Whereas, the water sample is directly subjected to serial dilution if required.
2. Pour the diluted samples onto the SOB agar plate and incubate at 30 °C for 24 h.
3. After completion of the incubation period, pick up properly well-defined colonies using wire loop and streak them on a new SOB agar plate.
4. Streak or spread inoculate the purified colonies on thiosulfate agar plate containing bromophenol blue.
5. Incubate the plates at 30 °C till the colonies are visible.
6. The pH reduction of sulfur-oxidizing bacteria from purple to colorless indicates positive.

3.4 Sulfate Ion Production Ability of SOB [11]

The screened bacterial isolate, which is found to reduce maximum pH, and colour of the broth medium, are further checked for their sulfate ion determination ability test. The amount of sulfate ion (SO_4^{2-}) produced during the growth of sulfur-oxidizing bacteria (SOB) on the thiosulfate broth medium are determined spectrophotometrically.

1. Separate SOB culture supernatant from the broth medium by spinning at $5488 \times g$ or /RCF for 10 min.
2. Add barium chloride (10% w/v) to the culture supernatant in the proportion of 1:1 followed by vigorous mixing.

3. The entire solution system becomes turbid (white turbidity) due to the formation of barium sulfate and measure the resulted turbidity at 450 nm with spectrophotometer.
4. Prepare sulfate standard curve/calibration curve using potassium sulfate (K_2SO_4). Prepare standard sulfate solution by dissolving the K_2SO_4 in deionized water to get the final concentrations in the range of 0–3 mM.
5. Compare the observed value with the sulfate standard calibration curve. The amount of turbidity formed is proportional to the sulfate concentration.

3.5 Identification of Sulfur-Oxidizing Bacteria Using 16S rRNA Gene Sequencing Methodology [12]

1. Inoculate the screened pure culture into 50 mL of thiobacillus medium and incubate overnight for its required growth.
2. Transfer an aliquot of 10 mL of overnight freshly grown culture to eppendorf tube and centrifuge at 4000 g for 10 min.
3. Discard the supernatant and re-suspend the pellet with 1 mL phosphate buffer saline (1×) and centrifuge at 4000 g for 10 min.
4. Discard the supernatant and add 0.5 mL 1× Tris EDTA buffer (pH 8.0).
5. Place the tube in the dry bath at 95 °C for 10 min and transfer immediately to −20 °C and perform DNA isolation using phenol-chloroform extraction method.
6. Perform standard PCR to amplify the 16S rRNA gene for the identification of the isolate. Prepare the PCR reaction (25 µL) by adding 2.5 µL 10× PCR buffer, 0.5 µL dNTP, 1.5 µL $MgCl_2$, 1 µL forward primer, 1 µL reverse primer, 0.2 µL 5 U Taq polymerase, 16.8 µL sterile distilled water, and 2.5 µL template DNA.
7. Place the PCR tubes in thermal cycler by adjusting the cycle condition to 94 °C for 2 min initial denaturation, 35 cycles of 94 °C for 30 s denaturation, 50 °C for 60 s annealing and 68 °C for 90 s extension and a final extension at 68 °C for 7 min.
8. Perform gel electrophoresis to check amplified PCR product along with standard DNA ladder.
9. Purify the 16S rRNA gene and subject for the sequencing using available sequencing platform.

3.6 Phylogenetic Analysis of the Isolates [13]

1. The sequence is mapped to the nucleotide-nucleotide BLAST (Blastn) database (<http://www.ncbi.nlm.nih.gov/BLAST>) and also check for the Seqmatch program and CHIMERA CHECK program of the Ribosomal Database Project (<http://rdp.cme.msu.edu/>).
2. Retrieve the subject sequences, and their closest matches from the database by alignment using the software Clustal X.

3. Construct the phylogenetic tree by the neighbor-joining method with the software MEGA. Calculate the evolutionary distances of nucleotide sequences with Kimura's 2-parameter model.
4. The calculated taxonomic distance is the key parameter to classify the bacteria at the appropriate taxonomic levels based upon some following stringent criteria.
5. The sequences with identity scores greater than 97% identity (<3% divergence) are considered to be resolved at the species level, between 95 and 97% at the genus level, between 90 and 95% at the family and between 85 and 90% at the order level, 80 and 85% at the class and 77–80% at phyla level.

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Isolation of Ammonia Oxidizing Bacteria

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Abstract

Ammonia oxidizing bacteria (AOB) oxidize ammonia to nitrate and play significant role in nitrogen cycle. Isolation and cultivation of AOB poses difficulty due to their complex requirement and slow growth rate, hence, culture independent methods are widely applied to study their presence in various ecosystems. In order to investigate basic physiology and to explore the potential of AOB, batch cultivation in laboratory is also necessary. Techniques for enrichment and isolation of AOB on solid media and in liquid media, like serial dilution method and dilution to extinction method, are discussed in the present chapter. To check the presence of AOB during their growth period, various parameters are monitored like utilization of ammonia, production of nitrate and/or nitrite, and presence of heterotrophs. Methods for their qualitative and quantitative estimation are also incorporated in the chapter.

Key words Ammonia oxidizing bacteria (AOB), Isolation, Ammonia oxidation, Dilution to extinction, Enrichment

1 Introduction

Nitrogen, the most abundant gas present in the normal air, is provided to plants and animals through biogeochemical nitrogen cycle via a process called nitrification. Nitrification process consists of two stepwise reactions: (a) ammonia oxidation and (b) nitrite oxidation. In first ammonia oxidation step ammonium (NH_4) is oxidized to nitrite (NO_2) by ammonia oxidizer microorganisms including ammonia oxidizing bacteria (AOB) or ammonia oxidizing archaea (AOA). They generate energy solely by the oxidation of ammonia to nitrite. Second step, nitrite (NO_2) oxidation to (NO_3) is carried out by nitrite oxidizing bacteria (NOB) [1].

Based on phylogenetic diversity study, five genera of AOB have been recognized and categorized in the Proteobacteria class. Most of the AOB, like *Nitrosomonas* and *Nitrospira*, belong to *Betaproteobacteria* while few belong to *Gammaproteobacteria* [2].

Cultivation of AOB through conventional culture techniques is difficult due to their complex requirement and slow growth rate. Culture independent molecular methods like Polymerase Chain Reaction (PCR), Fluorescence In situ Hybridization (FISH), Denaturing Gradient Gel Electrophoresis (DGGE), Terminal Restricted Fragment Length Polymorphism (T-RFLP), quantitative PCR (qPCR), and quinone profile are widely applied to get insight of environmental community composition and abundance. For identification of AOB by molecular methods, 16SrRNA or *amoA* (the gene encodes the alpha subunit of enzyme used in oxidation of ammonia—ammonia monooxygenase) is targeted.

AOB can be cultivated in laboratory by batch and continuous culture methods. Batch culture is easy for cultivation as compared to continuous culture. In this chapter, we have described various commonly used AOB isolation techniques by using solid media or liquid media in batch culture. To increase the number of AOB, enrichment is carried out by liquid media. Isolation by solid media is carried out on media solidified by agar or gellan gum. Liquid media are used for isolation by serial dilution or dilution to extinction method. Various chemical estimations, required as a part of monitoring of the cultivation, are also described.

2 Materials

2.1 Sample

1. Water (Fresh water, marine water, brackish water, etc.).
2. Fertilized soil, soil sample from mangrove ecosystem, rhizospheric soils.
3. Soil and effluent of fishpond with/without biofilters.
4. Seed sludge from the nitrification stage of a municipal wastewater treatment plant.
5. Nitrifying granules from aerobic upflow fluidized bed (AUFB) reactor.
6. Sewage from aeration tank.

2.2 Media

For enrichment or growth of AOB and AOA, media is generally supplied with either NH_4Cl or $(\text{NH}_4)_2\text{SO}_4$ as the ammonium source. Concentration of the ammonium salt may vary with the nature of sample and physiological state of the organisms while the rest of the general components remain same in most of the cases. pH maintenance of the media is done by various buffers. Usually, phosphate buffer is widely used but sometime as an alternative HEPES buffer can also be used because many AOB are sensitive to high concentration of phosphates.

2.2.1 Modified Alexander (MA) Medium: [3]

Liquid MA Media:
Composition (per Liter)

Ingredients	Amount
$(\text{NH}_4)_2\text{SO}_4$	2 g
K_2HPO_4	0.5 g
NaHCO_3	0.5 g
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.050 g
$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	0.005 g
$\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$	0.002 g
Fe-EDTA(III)	0.005 g
$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$	0.1 mg
$\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$	0.005 mg
$\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$	0.000 mg
$\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$	0.1 mg
HEPES (pH 7.8)	11.92 g (50 mM)
pH	7.8

1. Dissolve all the components, except K_2HPO_4 , in distilled or deionized water and make the final volume 900 ml. Separately dissolve 0.5 g K_2HPO_4 in water and make the final volume 100 ml. Adjust the pH of media.
 2. Autoclave the media at 121 °C for 15 min.
 3. After autoclaving, mix sterilized K_2HPO_4 with rest of the media aseptically to make final volume 1000 ml.
- Solid MA Media
1. Solid MA medium is prepared by adding 10 g Gellan gum to 1 l liquid MA medium.

2.2.2 Mineral Salt Medium

For preparation of mineral salt medium, researchers have proposed media with different chemical components and concentration. Here, we have described widely used media used for enrichment and cultivation of AOB.

Liquid Mineral Salt Medium

Components		Media A [4]	Media B [5]	Media C [6]
Solution MSM1	(NH ₄) ₂ SO ₄	0.330 g	X mM	–
	NH ₄ Cl	–	–	0.535 g
	MgSO ₄ · 7H ₂ O	0.040 g	0.049 g	0.049 g
	CaCl ₂	0.020 g	0.147 g	0.147 g
	NaCl	0.500 g	0.585 g	0.584 g
	KCl	–	0.075 g	0.074 g
	Bromothymol blue solution (0.04%)	5 ml	1 ml	–
	Cresol red solution (0.05%)	–	–	2 ml
	Trace elements solution	1 ml	1 ml	1 ml
	HEPES buffer	–	4 X mM	–
	Make the final volume with distilled water	900 ml	900 ml	900 ml
Solution MSM2	KH ₂ PO ₄	0.200 g	0.054 g	0.054 g
	Make the final volume with distilled water	100 ml	100 ml	100 ml
Autoclave solution MSM1 and MSM2 separately, mix both the solutions aseptically after sterilization to make the final volume 1000 ml. (Alternatively, solution MSM2 can be also filter sterilized)				
Adjust the pH (after sterilization)		7.5	7.8	7.8

1. Dissolve all the ingredients mentioned in the above table in distilled/deionized water to prepare solution MSM1 and MSM2.
2. Autoclave both the solutions separately at 121 °C for 20 min.
3. Allow it to cool, mix both the solutions MSM1 and MSM2 aseptically to achieve the final volume 1000 ml.
4. Adjust the desired pH of media by using sterilized solutions NaOH/Na₂CO₃/HCl/CH₃COOH.

Solid Mineral Salt Media

1. In distilled/deionized water, dissolve all the ingredients of Solution MSM1 mentioned in the above table (exclude dye solution), add 10 g agar powder to solution MSM1 and make the final volume 900 ml.
2. Prepare solution MSM2 as per above table.
3. Autoclave both the solutions (MSM1 and MSM2) separately at 121 °C for 20 min.
4. Mix both the solutions MSM1 and MSM2 aseptically to achieve the final volume 1000 ml.

- Adjust the desired pH of media by using sterilized solutions NaOH/Na₂CO₃/HCl/CH₃COOH. (Care must be taken that during this time media should not get solidified.)
- Distribute the media in pre-sterilized glass petridish.

2.3 Bromothymol Blue Solution (0.04%)

- Dissolve 0.04 g Bromothymol Blue in little amount of distilled water and make the final volume 100 ml with distilled water.

2.4 Cresol Red Solution (0.05%)

- Dissolve 0.05 g Cresol Red in little amount of distilled water and make the final volume 100 ml with distilled water.

2.5 Trace Element Solution

Components	For Media A [4]	For Media B [5]	For Media C [6]
Na ₂ EDTA	4.3 g	4.292 g	–
FeCl ₂ · 4H ₂ O	2 g	1.988 g	–
FeSO ₄ · 7H ₂ O	–	–	0.937 g
MnCl ₂ · 2H ₂ O	0.1 g	0.081 g	–
MnSO ₄ · 2H ₂ O	–	–	0.045 g
NiCl ₂ · 6H ₂ O	0.024 g	0.024 g	–
CoCl ₂ · 6H ₂ O	0.024 g	0.024 g	–
CuCl ₂ · 2H ₂ O	0.017 g	0.017 g	–
CuSO ₄ · 5H ₂ O	–	–	0.025 g
ZnCl ₂	0.068 g	0.068 g	–
ZnSO ₄ · 7H ₂ O	–	–	0.044 g
Na ₂ MoO ₄ · 2H ₂ O	0.024 g	0.024 g	–
(NH ₄) ₆ Mo ₇ O ₂₄ · 4H ₂ O	–	–	0.037 g
Na ₂ WO ₄ · 2H ₂ O	0.033 g	0.033 g	–
H ₃ BO ₃	0.062 g	0.062 g	0.049 g
1 M HCl	–	–	25 ml
Make final volume with distilled water	1000 ml	1000 ml	1000 ml

- Dissolve all the above mentioned ingredients in distilled water and make the final volume 1000 ml.
- Sterilize the solution by autoclaving at 121 °C for 15 min or by using 0.22µm filter.
- Store trace elements solution in dark at 4 °C in brown bottle.
- The trace elements solution can be stored and used for several years.

2.6 Contamination Test Media

Various low carbon concentration media are used to investigate the contamination of heterotrophs in AOB cultures.

Composition (per liter, pH 7.0 ± 0.2).

2.6.1 Medium 1: (Luria Broth)

Ingredients	Amount
NaCl	10 g
Tryptone	10 g
Yeast extract	5 g

2.6.2 Medium 2

Ingredients	Amount
Yeast extract	0.5 g
Beef extract	3 g
Peptone	5 g

2.6.3 Medium 3

Ingredients	Amount
NaCl	0.5 g
Yeast extract	5 g
Tryptone	20 g

2.6.4 Medium 4

Ingredients	Amount
NaCl	0.01 g
Yeast extract	0.1 g
Tryptone	0.4 g

1. Dissolve all the ingredients in water and make the final volume 1000 ml and adjust the pH.
2. Distribute 5 ml media per test tube.
3. Sterilize the media by autoclaving at 121°C for 15 min.

3 Methods

3.1 Enrichment of AOB

Enrichment of AOB is carried out in the same liquid media as growth/cultivation media but the initial ammonium concentration may be varied up to 20 mM.

1. Prepare liquid MA medium or any of the mineral salt medium described above.
2. Distribute 50 ml media in 125 ml Erlenmeyer flasks with cotton plug.
3. Aseptically, inoculate sample (usually 1 g soil/sediment or 1–5 ml water/sewage/effluent etc.) in liquid media.
4. After inoculation, remove some sample for measurement of initial concentration of NH_4^+ , NO_2^- , and NO_3^- .
5. Incubate the flask in dark, on mechanical shaker (optional). Incubation temperature is decided on the basis of environmental temperature of the sample. (e.g., for the samples from hot springs temperature is kept high, for mesophilic samples temperature is kept moderate, and for samples from cold environment temperature is kept low)
6. Monitor concentration of NH_4^+ , NO_2^- , and NO_3^- by sampling at regular intervals of 3–7 days. When around 70–80% of NH_4^+ is consumed, 5 ml of sample is transferred to 45 ml fresh media.
7. Repeat the above cycle for 3–5 times until the reproducible growth of culture is observed.

3.2 Isolation

Isolation is carried out from successful enrichment culture. Due to slow growth of AOB, difficulties arise in isolation. Many isolation procedures described by various researchers are broadly divided into isolation on solid media and isolation in liquid media.

3.2.1 Isolation by the Use of Solid Media

For isolation on solid media, AOB culture must be adapted to grow higher ammonium concentration (5 mM or higher), otherwise colonies will be small in size. Small sized colonies are difficult to pick and for further study. In order to prevent this problem, enrichment culture adapted at high ammonia concentration is used for isolation purpose.

Preparation of Serial Dilution of the Enrichment Culture

1. Standard serial dilution of enrichment culture is carried out up to final cell concentration 10^{-6} (10^{-1} to 10^{-6} ; total 6 tubes) by using respective liquid media as diluent (without addition of dye). (If researcher wish to use solid MA media for isolation, use liquid MA media as diluent. The same is true for mineral salt media.)
2. This diluted culture is used for isolation by spread or pour plate method.

Isolation by Spread Plate Technique

1. Take 0.1 ml aliquots from each tube and spread it on solid media without dye (which is same as liquid media used in serial dilution method) aseptically by glass spreader.

2. Incubate the plates, after closing with parafilm to prevent drying, for up to 3 months. Incubation temperature is decided by the environmental temperature of the sample.
3. Regularly check for the formation of the visible distinct colonies.

Isolation by Pour Plate Technique

1. 90 ml solution MSM1 of liquid mineral salt media, without indicator dye, is prepared with higher ammonium concentration and 1.5 g agar powder is added to it in a flask.
2. Prepare 10 ml solution MSM2 of liquid mineral salt media in another flask. Prepare at least 6 sets (Solution MSM1 and Solution MSM2) of flasks.
3. Sterilize all the flasks by autoclaving, after sterilization mix solution MSM1 and MSM2 aseptically.
4. Allow all the flasks of the mixed solutions to cool at 40–50 °C. Label them as 10^{-1} to 10^{-6} .
5. Add 1 ml of enrichment culture from serially diluted cultures test tube to respective flask. Mix gently, pour the media in four to five pre-sterilized petri dishes from each flask. Allow them to solidify and pack them with parafilm to prevent from drying.
6. Incubate them for up to 3–6 months. Incubation temperature is decided by the environmental temperature of the sample. Check the plates regularly for the formation of the visible distinct colonies.
7. Prepare liquid mineral salt medium with indicator dye and distribute them in test tube.
8. Pick each of the colony and transfer them into individual test tube containing liquid mineral salt media.
9. Incubate the test tubes for 4 weeks, remove samples and test them for NH_4^+ consumption and NO_2^- production.
10. Transfer the cultures that consumed around 70–80% or more of the NH_4^+ to fresh liquid mineral salt medium.
11. Test the cultures that do not use 80% of the NH_4^+ , incubate them again for 2 weeks up to 3 months and transfer them too when they consumed 80% or more of the NH_4^+ .
12. Test the transferred cultures again for NH_4^+ consumption and/or NO_2^- production after 2–4 weeks of incubation and transfer if necessary.
13. During the third transfer, inoculate the culture into contamination test media to check for contamination with heterotrophic bacteria. For contamination checking, inoculate loopful of

culture into contamination test media aseptically and incubate the tubes for at least 3–4 weeks. Cultures that show heterotrophic contamination should be omitted.

14. Transfer non-contaminated cultures to 50 ml fresh liquid mineral salt media in 125 ml Erlenmeyer flask and incubate it.
15. Remove the sample at regular interval to check the NH_4^+ concentration, when around 80% of the NH_4^+ is used, filter the culture in the Erlenmeyer flask through a $0.2\mu\text{m}$ nitrocellulose filter for molecular analysis to identify the isolated strain. Filters should be stored at -20 or -80°C .

3.2.2 Isolation by the Use of Liquid Media

Dilution to Extinction

Method: [7]

Stage 1: Preparation of Extinction Dilution

1. Take 1 g of soil or 1 ml of liquid sample, mix it with 9 ml with sterile liquid mineral salt media B to achieve dilution 0.1 g/ml (Dilution of sample: 10^{-1}) and mix well (Homogenizer can be also used for mixing purpose).
2. Check the pH after mixing, and adjust pH same as of original liquid mineral salt media.
3. Transfer $100\mu\text{l}$ diluted sample to 4.9 ml liquid mineral salt media test tube. (Dilution of sample: 5×10^{-2}).
4. Transfer $500\mu\text{l}$ from above tube to 4.5 ml liquid mineral salt media test tube (Dilution of sample: 5×10^{-3}). Repeat the same procedure up to 5×10^{-8} . For each sample, prepare such two series.
5. Wrap the tube with parafilm, and incubate in the dark for 3–4 months. Incubation temperature is decided on the basis of environmental temperature of the sample.
6. Growth is detected by color change in the medium, sample is also tested for the production of nitrite by spot test by modified Griess Ilosvay reagents [8].

Stage 2: Starvation of Heterotrophic Organisms and Isolation of AOB

1. Prepare mineral salt media B without HEPES. Pour 20 ml media in 120 ml serum bottle. (Prebake serum bottles at 400°C for 2 h for removal of any possible organic material which can support growth of heterotrophic organisms. The bottles are sealed with Teflon-faced silica septa as some of the AOB are sensitive to rubber butyl septa.)
2. Transfer $100\mu\text{l}$ of culture from highest extinction dilution AOB positive culture to serum bottle.
3. Incubate inoculated bottles in reciprocal shaker at 100 rpm at temperature decided on the basis of the sample. Incubate the culture till color change occurs.
4. Then, remove the sample by sterile syringe to check the concentration of NH_4^+ , NO_2^- , pH, and total number of cell by microscopic cell counting chamber or alternatively by acridine orange direct count AODC [9]. Also check contamination of

heterotrophic bacteria by dilution plating method on solidified contamination media.

5. On the basis of total number of cells, dilute cultures in liquid media 50 cells/ml. Use this dilution for inoculation of 96 well sterile microtiter plate.
6. Inoculate 5, 20, and 100 μ l to give 0.25–5 cells/well respectively. Adjust volume 250 μ l per well by adding liquid media.
7. Wrap the microtiter plate with parafilm, and incubate in dark until the expected color change is observed. Check the presence of heterotrophic organisms by inoculating 5–10 μ l of culture from well to contamination test media, wrap with parafilm and incubate in dark for several weeks at room temperature.
8. Select the culture with AOB positive but heterotrophic organism negative, and transfer it high volume of fresh liquid media.

Method for isolation by dilution to extinction of AOB at low ammonium concentration is described by Bollmann et al. [5]. This method is preferred when researcher wish to isolate AOB at very low ammonium concentration.

1. Grow the culture in enrichment media until around 70–80% of NH_4^+ is consumed.
2. Remove the sample for counting the cell number in sample. Perform the staining with 4',6-diamidino-2-phenylindole (DAPI) [10] and count the cell number under epifluorescence microscope or count the cell number by microscopic cell counting chamber.
3. Dilute the culture with 100 ml liquid mineral salt media (NH_4^+ concentration same as enrichment media) to make a final concentration 2.5 cells/ml.
4. From above diluted culture transfer 200 μ l of culture per well in 96-well microtiter plate. Incubate the plate, after wrapping with parafilm, in dark.
5. After incubation, to another 96-well microtiter plate transfer 10 μ l of culture from each well of previous microtiter plate to determine nitrite.
6. Transfer positive AOB cultures to 50 ml fresh liquid mineral salt media in 125 ml Erlenmeyer flask and incubate it.
7. Remove the sample at regular interval to check the NH_4^+ concentration, when around 80% of the NH_4^+ is used, filter the culture in the Erlenmeyer flask through a 0.2 μ m nitrocellulose filter for molecular analysis to identify the isolated strain. Filters should be stored at -20 or -80 $^{\circ}\text{C}$.

Serial Dilution Method

1. Take 96 (8 well $\times$ 12 row) well microtiter plate to carry out MPN dilution. Add 100 μ l of liquid mineral salt media to each (96) well. Then, add 100 μ l sample to first 8 wells (First row).
2. Perform serial dilution by transferring 100 μ l of mixture content from each well to its corresponding well in next row. Repeat the same procedure across the plate. At the end of process, sample is diluted 12 two-fold dilution with eight replicates.
3. Cover the plate with parafilm, incubate the plate at specific temperature (decided on the basis of environmental temperature of the sample) for at least 2–3 months.
4. After incubation, transfer 10 μ l per well to another 96 (8 well $\times$ 12 row) well microtiter plate. Determine the concentration of nitrite from that.
5. Use highest positive dilution for another dilution series. Repeat this procedure for two to three times.
6. Transfer positive samples from highest positive dilution tube to fresh liquid mineral salt media and determine its purity by using contamination test media.
7. Determination of NH_4^+ , nitrite, and nitrate.

3.2.3 Supplementary Methods

Sequencing of DNA

Sample: *Pure colonies on solid media or pure culture in liquid media. For pure culture in liquid media, filter 20–50 ml of culture through 0.1–0.2 μ m nitrocellulose paper and transfer it to screw capped bottle, store it at -20 to -80°C freezer. Carry out bead beating process, by adding 500 μ l of high salt buffer (1 M NaCl or 5 mM MgCl_2 or 10 mM Tris, pH 8) to that bottle. Also add, 1 g of silica or zirconia beads. Bead beat this tube for 30 s. Now, cool it on ice for 15 min. Then again bead beat the tube. Repeat this process for three cycle.*

DNA isolation: *Carry out DNA isolation and extraction by DNA isolation kits as per manufacturer instruction or by standard phenol/chloroform method.*

Amplification of *amoA* and 16S rRNA gene: *Monooxygenase is key enzyme produced by AOB and AOA. Gene encoding the structural subunits of this enzyme are used to identify AOB and AOA. Primers for various subunits are available but most researchers use primers targeting *amoA* gene. Various primers used in identification are deeply described by Bollmann et al. [5]*

Determination of Concentration of NH_4^+ [11]

Reagents

1. **NaOH Solution:** 12 g/l in water.
2. **Sodium nitroprusside reagent:** Dissolve 85 g of sodium salicylate and 0.6 g of sodium nitroprusside in 900 ml and water and make the final volume 1000 ml.
3. **Sodium dichloroisocyanurate:** 0.2 g/l in water.

4. **10 mM NH_4^+ calibration stock solution:** 0.6607 g/l $(\text{NH}_4)_2\text{SO}_4$.
5. Store all above solution at 4 °C.
6. **200 μM NH_4^+ calibration working solution:** 200 μM NH_4^+ by diluting stock calibration solution in a volumetric flask. Dilute it further to 10, 25, 50, 100, and 200 μM NO_2^- .

Determination By Using 48-Well Plates

1. Add 500 μl of sample in a well.
2. Prepare 2:1 sodium hydroxide: sodium nitroprusside reagent freshly. Add 250 μl of this reagent to sample and mix it.
3. Add 100 μl of sodium dichloroisocyanurate solution and mix the content by shaking the plate gently.
4. Incubate the plate in dark for 30 min.
5. Measure the absorbance at 660 nm by plate reader by using water as blank.
6. Prepare the standard curve of ammonium by replacing sample with working calibration solution.

Determination By Using Test Tube

1. Take 2 ml sample in test tube.
2. Add 1 ml of freshly prepared 2:1 sodium hydroxide: sodium nitroprusside reagent.
3. Add 0.4 ml sodium dichloroisocyanurate, mix it well.
4. Incubate in dark for 30 min.
5. Measure the absorbance 660 nm against water as blank.
6. Prepare standard graph by replacing sample with working calibration solution of NH_4^+ .

Determination of NO_2^- [12]

Reagents

1. **Griess Reagent:**
 - (a) Prepare diluted phosphoric acid by adding 100 ml (85%) phosphoric acid in 750 ml of water. Dissolve 10 g/l sulfanilamide (the solution might need to be heated slightly to dissolve the sulfanilamide), add 0.5 g/l *N*-(1-naphthyl) ethylenediamine (light sensitive, store in foil covered bottle).
2. **NO_2^- calibration stock solution (10 mM):** Prepare 0.851 g/l KNO_2 or 0.69 g/l NaNO_2 in MilliQ water.
3. Store above all the reagents at 4 °C.
4. **NO_2^- calibration working solution:** 100 μM NO_2^- by diluting stock calibration solution in a volumetric flask. Dilute it further to 10, 25, 50, and 100 μM NO_2^- .

Determination of the NO_2^- Concentration by Using the Plate Reader (48-Well Plates)

1. Add 500 μl of sample and add 125 μl of the Griess Reagent and shake the plate.
2. Incubate the plate in dark for 10 min.
3. Measure the absorbance in the plate reader at 540 nm by using water as a blank.
4. Prepare standard curve for NO_2^- by replacing sample with working calibration solutions.

Determination of the NO_2^- Concentration by Using the Plate Reader (96-Well Plates) to Screen Isolation Experiments

1. Take a new 96-well plate and transfer 10 μl of sample from a 96-well plate (AOB culture) using 8-channel pipette.
2. Add 90 μl of water to each well.
3. Add 20 μl of Griess reagent to each well.
4. Incubate the plate in dark for 10 min.
5. Measure the absorbance in the plate reader at 540 nm by using water as a blank.
6. Prepare standard curve for NO_2^- by replacing sample with working calibration solution.

Spot Test for Nitrite Detection Using Modified Griess Ilosvay Reagent [8]

1. Reagents:
 - (a) Diazotizing reagent: 0.5 g% sulfanilamide in 2.4 M HCl, store in refrigerator. Handle with care due to its carcinogenic potential.
 - (b) Coupling reagent: 0.3 g% *N*-(1-naphthyl)ethylenediamine in 0.12 M HCl. Reagent is light sensitive, store in dark bottle in refrigerator.
2. Procedure:
 - (a) Take 0.1 ml of sample in spot plate.
 - (b) Add one drop of diazotizing reagent and then one drop of coupling reagent.
 - (c) Color production (pink to red) indicates that NO_2^- is present.

Determination of (NO_2^- plus NO_3^-) Determination [13]

1. **Catalyst Solution:** Dissolve 35.4 mg of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ and 900 mg of $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ in 900 ml distilled water and make the final volume 1000 ml with distilled water.
2. **1 N NaOH:** Dissolve 40 g NaOH in 900 ml distilled water and make the final volume 1000 ml.
3. **Hydrazine sulfate solution:** Dissolve 1.71 g/l hydrazine sulfate in distilled water.
4. **Sulfanilamide solution:** Prepare 3.5 M HCl by diluting conc. HCl. Dissolve 10 g sulfanilamide in 900 ml 3.5 M HCl and make the final volume 1000 ml with 3.5 M HCl.

5. **NEDD (Naphthylethylene diamine dichloride) solution:** Prepare 1 g/l NEDD in water. (store in dark bottle)
6. **10 mM NO_3^- stock calibration solution:** Prepare 1.011 g/l KNO_3 or 0.8499 g/l NaNO_3 in water.
7. Store above all solutions in refrigerator at 4 °C.
8. **100 μM NO_3^- working calibration solution:** Dilute stock calibration solution to make 100 μM NO_3^- in volumetric flask. Dilute it further to make 25, 50, 75, and 100 μM NO_3^- .

Determination of the (NO_2^- plus NO_3^-) Concentration by Using the Plate Reader (48-Well Plates)

1. Take 48-well plate, add 135 μl of sample.
2. Add 20 μl of catalyst solution and shake the plate well.
3. Add 20 μl of NaOH solution and shake the plate well.
4. Add 20 μl of hydrazine sulfate solution and shake the plate well.
5. Incubate the plate 15 min at room temperature.
6. Add 75 μl sulfanilamide solution and shake the plate.
7. Add 20 μl NEDD solution and shake the plate well.
8. Incubate for 10 min.
9. Measure the absorbance at 540 nm by plate reader by using water as blank.
10. In the similar way, prepare standard curve by replacing sample with working calibration solution.

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Isolation and Identification of Nitrite-Oxidizing Microbes

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Abstract

Nitrite-oxidizing bacteria (NOB) are Gram-negative microorganisms, which pick up their energy by changing nitrite over to nitrate. Nitrification is a microbially catalyzed process whereby ammonia (NH_3) is oxidized to nitrite (NO_2^-) and thusly to nitrate (NO_3^-). NOB convey the second phase of the nitrification cycle, that of oxidation of nitrite to nitrate. Since the microorganisms liable for nitrification are fundamentally autotrophic, nitrification gives an interesting connection between the carbon and nitrogen cycles. An unadulterated culture of a nitrite-oxidizing bacterium can be gotten by density gradient centrifugation and a conventional dilution technique. For the enrichment of predominant nitrifiers, combination of the most probable number technique followed by the serial dilutions strategy is recommended. In the highest dilutions, nitrite oxidation can be distinguished simply following a while as a result of the slow growth of the nitrite oxidizers.

Key words Autotrophic, Nitrite, Nitrate, Nitrification, Oxidation

1 Introduction

Nitrite-oxidizing bacteria (NOB) are a small group of essentially organo as well as chemoautotrophs that are hard to grow and work with. NOB give a critical connection in the global nitrogen cycle between the ammonia-oxidizing microbes, which produce nitrite, and the different denitrifying microorganisms that eliminate nitrate by reducing it to ammonia or molecular nitrogen, accordingly completing the global nitrogen cycle. The key enzyme that makes the NOB so significant in the global nitrogen cycle is nitrite oxidoreductase (NOR). Nitrite oxidation is a strictly aerobic cycle and all C-requirements can be met from assimilation of CO_2 [1].

The nitrifying cycle in a microorganism happens in two stages: (1) oxidation of ammonia to nitrite and (2) oxidation of the nitrite to nitrate. Microorganisms of various genera and species participate in every one of these stages; the microbes species that can straightforwardly change ammonium over to nitrate have not yet been distinguished. Nitrite oxidizers are fit for reducing nitrate to nitrite

under anaerobic conditions. Obligate and facultative lithoautotrophic nitrite oxidizers can be effectively isolated just if mineral nitrite media liberated from any natural impurities are utilized, in any case heterotrophs will congest nitrite oxidizers. The oxidation is done by microorganisms of the genera *Nitrosomonas* sp., *Nitrosococcus* sp., *Nitrosospira* sp., *Nitrosolobus* sp., and *Nitrosovibrio* sp.

2 Materials

Sample can be different soil types, farmyard manure, and aerated sewage effluent.

2.1 Dye Based Isolation of NOB

1. Enrichment Media: Nakos and Wolcott (1979) medium (g/L): 0.3 (NH₄)₂SO₄; 0.136 CaCl₂; 0.175 MgSO₄ · 7H₂O; 0.5 NaHCO₃; 13.5 Na₂HPO₄; 0.7 KH₂PO₄; 0.005 FeSO₄ · 7H₂O and 0.00375 NaMoO₄, pH 8.2 [2].
2. Culture Media: 0.006 NaNO₂; 1.0 K₂HPO₄; 0.3 NaCl; 0.1 MgSO₄ · 7H₂O; 0.03 FeSO₄ · 7H₂O; 0.3 CaCl₂; and 1.0 CaCO₃. Add pH indicators (Methyl orange, Bromocresol green, Methyl Red, Bromothymol blue, and Phenol red) to the enumeration media before sterilization. Distribute the 5 mL portions in tubes and plugged it. The pH of the media should be adjusted to 7 with 0.01 mol/L NaOH. After sterilization, set the pH of the media to 8.2 by the addition of sterile NaOH [3].

2.2 Percoll Based Isolation of NOB

1. Mineral medium: 0.5 g NaNO₂; 0.007 g CaCO₃; 0.5 g NaCl; 0.05 g MgCl₂ · 6H₂O; 0.15 g KH₂PO₄; 33.8 mg MnSO₄ · H₂O; 49.4 mg H₃BO₃; 43.1 mg ZnSO₄ · 7H₂O; 37.1 mg (NH₄)₆ Mo₇O₂₄ · 4H₂O; 25.0 mg CuSO₄ · 5H₂O; 973.0 mg FeSO₄ · 7H₂O. 1000 ml distilled water, pH 8.6 [4].
2. 1.5 M NaCl.
3. Percoll.

3 Methods

3.1 Dye Based Isolation of NOB

1. Prepare tenfold dilution series of soils, manure, sewage, or effluent samples in sterile d/w.
2. Inoculate the samples in tubes containing media for 5 appropriate successive dilutions. Incubated all tubes for 5–8 weeks at 30 °C. For the detection of positive samples, visually score the tubes at 2-day intervals for the production of sufficient acid to decrease the pH and thus change the color of the indicator added as an indication of nitrifier growth.

3. It requires lengthy incubation periods (weeks) due to the slow growth of nitrifying bacteria.

3.2 Percoll Based Isolation of NOB

1. Enrichment Step: Add Mineral nitrite medium (100 mL) to a sample and incubate in Erlenmeyer flask at 37 °C in the dark.
2. Adjust the nitrite concentration to 0.7–1.4 mM. Add sterile nitrite from a stock solution to the starting concentration. Enrich the nitrite oxidizer further by density gradient centrifugation.
3. Centrifuge the cells and resuspend in 1 mL 0.9% NaCl. Mix cell suspension with 56 mL Percoll and 24 mL 1.5 M NaCl solution and centrifuge in 10-mL tubes for 2 h at $12,000 \times g$ and 4 °C.
4. Adjust the gradient density between 1.02 and 1.14 g/cm³.
5. One distinct brownish band will appear that contains the highest numbers of nitrite-oxidizing cells.
6. Remove the Percoll and resuspend the cells in 0.9% NaCl and serially dilute in mineral nitrite medium with 0.2 g NaNO₂/L and incubate for 10 weeks.

4 Note

1. NOB can endure starvation from energy and reductant for several years. Further, it can take a week to months until their metabolism will be reactivated and growth resumes. Consequently, cultures must be turned on prior to performing the experiments [5].

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Isolation and Identification of Iron-Oxidizing Microbes

Ami Naik and Pooja Patel

Abstract

Microbial oxidation of iron is an important part of biogeochemical cycles in mine environment. Iron-oxidizing bacteria are important catalysts of iron cycling in wetland environments. However, little is known about their diversity and distribution. Many bacterial species have been found in soil deposits but only a few pure cultures have been isolated. The aim of this protocol is to isolate iron-oxidizing bacteria from different sources. Here, we describe the procedure for isolation, identification, and characterization of iron-oxidizing bacteria. The classical approach for the cultivation of iron-oxidizing bacteria is agar-based gradient tube method where the bacteria find optimal growth conditions in opposing gradients of oxygen (O₂) and dissolved Fe (II). Two separate enrichment cultures are used, and in each, the cells grow in a distinct, rust-colored band in the gel at the oxic-anoxic interface. Another method is non-gradient, 9K medium method to determine the distinct growth characteristics of isolates.

Key words Iron oxidizing, Bacteria, Gradient plate, 9K medium, Environment

1 Introduction

Iron (Fe) is the fourth most abundant element of the lithosphere. This element participates in many life-sustaining geochemical and microbiological processes. Iron is an integrated molecule in many cellular compounds. It has a very high modern as well as ancient relevance and significance in environments. Iron is involved in biotic and abiotic redox process in environment [1]. In nature, this element found in two redox states, either oxidized ferric iron (FeIII) or as reduced ferrous iron (FeII). It is considered an essential micronutrient that takes part in many key biological processes, such as gene regulation, DNA biosynthesis, N₂ fixation, photosynthesis, oxygen transport, and many more [2]. Fe is crucial for growth and relatively abundant in the soil, though, its availability to plant roots under alkaline soil is very low than that required for an optimal growth of plants [3]. Therefore, nowadays iron deficiency is becoming common problem for many crops. To overcome this problem, Fe-oxidizing bacterium can serve as important

catalyst to cycling between states of iron through a variety of mechanisms, such as altering pH, sorption, and enzymatic conversion of iron to oxidized forms [4]. Plant and iron oxides coexist in soils, and therefore their close association provides abundant opportunities for mutual interactions with an impact on mineral weathering. Beside this, from a microbiological point of view, it is a challenging task to isolate Fe-oxidizing prokaryotes in laboratory by simple culture method. Concentration of iron and oxygen is found crucial to cultivate Fe-oxidizing bacteria; therefore gradient and 9K medium methods are highly recommended for the enrichment and identification of Fe-oxidizing bacteria. Iron-oxidizing species are mainly affiliated with the Proteobacteria and the in vitro evidence supporting their potential role in Fe-cycling [5].

2 Materials

Prepare all the media in distilled water. Adjust the pH of the media using H_2SO_4 or NaOH. If not noted, incubate all the liquid and solid media at 30 °C and 25 °C, respectively.

2.1 Gradient Tube Method

2.1.1 FeS Plug Preparation

1. Heat 300 ml of distilled water to 50 °C.
2. Simultaneously, take a separate beaker and heat another 300 ml of distilled water and keep aside.
3. Now, measure 46.2 g $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ and 39.6 g Na_2S .
4. Add the FeSO_4 to the beaker first, followed immediately by the Na_2S . Stir until all Na_2S has dissolved.
5. Decant into a clean 500 ml glass bottle. Rinse the first beaker with pre-warmed distilled water and fill the bottle to the neck. It is important to ensure that the equal molar amounts of the components are maintained.
6. Close the bottle with a rubber stopper.
7. The precipitate will begin to settle immediately. Allow the precipitate to settle completely before the first wash.
8. To wash, decant the supernatant carefully and add new distilled water to the neck of the bottle.
9. Repeat this wash procedure five times before use in culture propagation [6].

2.1.2 Modified Wolfe's Minimal Medium (MWMM)

Top Layer

- MWMM (see below).
 5 mM NaHCO_3 .
 1 μl Wolfe's Mineral Solution (see below) per 1 ml MWMM.
 1 μl Wolfe's Trace Solution (see below) per 1 ml MWMM.¹
 0.15% high melt agarose.

¹ Add vitamins aseptically after autoclaving.

Bottom Layer

MWMM.

FeS (see above).

1.0% high melt agarose.

Modified Wolfe's Minimal Medium

NH ₄ Cl	0.10 g
MgSO ₄ · 7H ₂ O	0.20 g
CaCl ₂ · 2H ₂ O	0.10 g
K ₂ HPO ₄	0.05 g
NaHCO ₃	20.0 mM
Thiosulfate	10.0 mM
Wolfe's Mineral solution (see below)	10.00 ml
Vitamin solution (see below)	10.00 ml
Agar	15.00 g
Distilled water	1.00 l

Adjust the pH of MWMM to 6.5–6.8 using mixture of N₂ and CO₂ gas. Dispense the medium under gas phase of 80% N₂ and 20% CO₂ into Balch tubes and autoclave. Add 10 µl/ml vitamins and mineral after autoclaving. For growth, the headspace is supplemented with 2–5% O₂.

Wolfe's Mineral Solution

Available from ATCC as a sterile ready-to-use liquid (Trace Mineral Supplement, catalog no. MD-TMS).

Nitrilotriacetic acid	1.5 g
MgSO ₄ · 7H ₂ O	3.0 g
MnSO ₄ · H ₂ O	0.5 g
NaCl	1.0 g
FeSO ₄ · 7H ₂ O	0.1 g
CoCl ₂ · 6H ₂ O	0.1 g
CaCl ₂	0.1 g
ZnSO ₄ · 7H ₂ O	0.1 g
CuSO ₄ · 5H ₂ O	0.01 g
AlK(SO ₄) ₂ · 12H ₂ O	0.01 g
H ₃ BO ₃	0.01 g
Na ₂ MoO ₄ · 2H ₂ O	0.01 g
Distilled water	1.0 l

Add nitrilotriacetic acid to approximately 500 ml of water and adjust to pH 6.5 with KOH to dissolve the compound. Bring volume to 1.0 l with remaining water and add remaining compounds one at a time.

Wolfe's Vitamin Solution Available from ATCC as a sterile ready-to-use liquid (Vitamin Supplement, catalog no. MD-VS).

Biotin	2.0 mg
Folic acid	2.0 mg
Pyridoxine hydrochloride	10.0 mg
Thiamine · HCl	5.0 mg
Riboflavin	5.0 mg
Nicotinic acid	5.0 mg
Calcium D-(+)-pantothenate	5.0 mg
Vitamin B ₁₂	0.1 mg
<i>p</i> -Aminobenzoic acid	5.0 mg
Thioctic acid	5.0 mg
Distilled water	1.0 l

2.1.3 Sodium Bicarbonate (NaHCO₃) (1 M) Dissolve 12.6 g of NaHCO₃ in 100 ml of H₂O.

2.2 Non-gradient Method (9K Medium) 0.3 g of (NH₄)₂SO₄.
0.01 g of KCl.

2.2.1 Media A 0.05 g of K₂HPO₄.
0.05 g of MgSO₄ · 7H₂O.
0.01 g of Ca(NO₃)₂.
Add in 100 ml of distilled water and this salt solution was autoclave the media.

2.2.2 Media B 1 g FeSO₄ · 7H₂O.
10 ml 1 N H₂SO₄.
Make up the total volume up to 100 ml using distilled water.
Sterilize media A by autoclave and sterilize B solution by using filter.
And mix both the solutions before use.

3 Methods

3.1 Soil Sampling (see Note 1)

1. Collect the crushed iron ore sample from the different locations of the iron ore mining sites from the upper layer of soil after removing top layer (about 1 cm). Most of the microbial activities take place in this region and hence, bacteria population is concentrated. Collect the sample using clean dry spatula and sterile polythene bag.
2. Small sample can be used for enrichment of iron-oxidizing bacteria, and other samples can be freeze dried and stored for future analysis.
3. For enrichment and isolation, take 50 g of sample in a conical flask containing 100 ml of distilled water.

3.2 Enrichment and Isolation (see Notes 2 and 3)

3.2.1 Gradient Method

1. For the initial enrichment, isolation, and subsequent maintenance of culture, use the gradient tubes with opposing gradient [7] of oxygen and iron sulfide.
2. Use 16 mm screw cap glass tube as a gradient system to prepare two layers of agarose. The tubes should have a plug of FeS overlaid with bicarbonate-buffered medium and a headspace of air.
3. Take 1.25 ml of FeS and modified Wolfe mineral medium [8] at a 1:1 ratio for the preparation of bottom layer in a flask and amend it with 1% (wt/vol) agarose.
4. In a separate flask, prepare the overlayer by adding 0.15% (wt/vol) agarose to the MWMM medium.
5. Autoclave both the flasks at 121 °C for 20 min.
6. Now, add 0.75 ml of molten FeS to a pre-sterilized empty tube and allow agarose to solidify to form a solid plug.
7. Add sodium bicarbonate (5 mM final concentration; 1 M sterile, stock solution) and 1 ml/l of vitamins (filter sterilized) to molten overlayer [9].
8. Now, pour 3.75 ml of this mixture on the surface of FeS Plug.
9. Next, flush the molten overlayer with sterile CO₂ for 1 min to reach a final pH of between 6.2 and 6.4.
10. Cap the tubes with butyl rubber or stopper and allow them to sit for 6–24 h prior to inoculation.
11. Now, inoculate the tubes after removing the caps by inserting a pipette tip containing 10–15 µl of inoculant into the gel on to FeS plug. Incubate the tubes in the dark with a constant temperature of 21 °C (±1 °C).

12. Observe the tubes after 4–6 days for discrete band of Fe (III) oxide in contrast to the diffuse deposition of oxides in abiotic controls [10].
13. Harvest the growth bands from the several growth tubes and centrifuge on PO₄ buffer. Place the harvested material in the vials containing anaerobic medium.
14. Confirm the presence of bacteria by microscopy and molecular analysis.

3.2.2 *Non-gradient Method*

1. Take media A and B and after sterilization, mix them well in a conical flask in sterile conditions.
2. Now add 1 ml of sample solution to the conical flask.
3. Plug the cotton wool on conical flask and incubate it for 7 days in incubator shaker at 37 °C.
4. After 1 week, streak one loopful of enriched media on the same media containing agar and incubate at 37 °C for 48 h to get pure culture of isolates.
5. After 3 days, observe the dark yellow colored powdery growth on the plates.
6. Subculture the isolates for many times to get pure culture.
7. Confirm the presence of bacteria by microscopy and molecular analysis.

3.3 *Identification and Characteristics of Bacterial Isolates*

3.3.1 *Morphological Characterization*

1. Record the colony morphology of the isolates after incubation.
2. Observe the overall shape, size, opacity, elevation, margin of the colonies of isolates [11].
3. Perform the gram staining and observe under microscope and record the cell shape and arrangement.

3.3.2 *Identification of Isolates*

1. Extract the genomic DNA of pure culture isolates as per modified Marmur's method [12] and estimate the DNA concentration by measuring absorbance at 260 nm (Quantitative analysis).
2. Check the DNA on agarose gel electrophoresis (Qualitative analysis).
3. 16S rRNA genes were amplified by PCR using universal primers (16s-AGAGTTTGATCCTGGCTCAG and 16s-GGTTCCTTGTTACGACTT).
4. Purify the 16s rDNA PCR product and perform electrophoresis for the product.
5. Perform the sequencing of purified rDNA PCR products using sequencer.

6. Use similarity rank from Ribosomal Database Project (RDP) and FASTA nucleotide database query to determine the degree of similarity.
7. Analyze the phylogenetic relations using the protocol of Yakhymov [13] and draw phylogenetic tree using MEGA5 software with Neighbor-Joining method [14].

4 Notes

1. Sample sites for the iron-oxidizing bacteria can be:
 - (a) Iron ore mining sites
 - (b) Iron scrape sites
 - (c) Manganese mining sites
 - (d) Sulfur and iron sulfide mining sites
 - (e) Acid mining sites
 - (f) Copper mining sites such as sulfuric PAD, oxidic PAD, sulfuric dump, PLS, and leaching heap
 - (g) Sewage treatment plants
 - (h) Groundwater from a basement tile drain
2. Always inoculate the gradient tubes after 24 h of preparation of FeS plug to facilitate the diffusion of Fe^{2+} into the top layer where bacterial growth occurs.
3. Different medium compositions with varied ferrous concentration and pH in media B should be used.

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Isolation and Identification of Phytin Mineralizing Microbes

Swati Patel and Pritesh Patel

Abstract

Phytin is a source of phosphate and it is strongly associated with other mineral ions forming phytate mineral complexes. These mineral complexes are hard to absorb by plants. Soil microorganisms employ various mechanisms such as proton production during ammonium assimilation, organic acid, phytase and phosphatase production for dissolution of phytin. It converts unattainable phytin to its simpler lower phosphate esters to be fascinated by plants. It replenishes the level of phosphate and reduces the need of chemical fertilizers with diminution of phosphate pollution in soil.

Key words Phytin mineralization, Organic acid production, Phytase, Phosphatase

1 Introduction

Foremost portion (30–70%) of the phosphorous in the soil is of organic phosphate [1] derived from plant, animal, and microbial residues [2]. It is hard to solubilize this type of organic phosphorus (P_0) and thus it is least accessible to the plants. Phytin ($C_6H_{17}NaO_{24}P_6$) is an organic phosphate chemically hexaphosphoric acid ester of the hexavalent alicyclic alcohol mesoinositol found in the soil. Phytin resembles phosphoric acid but it is significantly strong. It is found in different forms such as sodium phytate, calcium phytate, ferric phytate and sorbed in soil so strongly that it decreases its susceptibility to mineralization. Phytin is extremely resistant to basic hydrolysis and rather resistant to acids also. Rhizosphere inhabiting microorganisms have a potency to secrete organic acids or phosphomonoesterases like phytases (myo-inositol-hexakisphosphate-phosphohydrolases) or phosphatases which hydrolyze it into various lower phosphate esters of myo-inositol and other phosphates, easily available to the plants [3]. Wide variety of bacteria like *Klebsiella pneumonia*, *Pseudomonas*, *Alcaligenes*, *Bacillus* sp., *Escherichia coli*, *Citrobacter braakii*, *Raoultella* sp., *Enterobacter*, *Yersinia* sp., arbuscular mycorrhizal (AM) fungi—

Rhizophagus irregularis, *Rhizophagus intraradices* and other fungal species like *Aspergillus*, *Penicillium*, *Trichoderma*, *Mucor piriformis*, *Rhizopus oligosporus*, *Cladosporium* sp. *Fusarium* [4] and yeasts are known to produce phytases and phosphatases for phytin mineralization which have further role in plant growth promotion too. This bioinoculant mineralizes unavailable phytin and makes it obtainable to plants to consume it in metabolism and this way reduces amalgamation of other chemical fertilizers in soil.

2 Materials

2.1 Isolation of Phytin Mineralizing Organism

1. Phytin mineralizing (PM) media or phytate screening medium (PSM): Glucose 10 g, sodium phytate 4 g, calcium chloride (CaCl_2) 5 g, ammonium nitrate (NH_4NO_3) 0.5 g, potassium chloride (KCl) 0.5 g, magnesium sulfate (MgSO_4) 0.01 g, iron sulfate heptahydrate ($\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$) 0.01 g, manganese sulfate (MnSO_4) 0.01 g, agar 15 g, pH 7.0 [5].
2. National Botanical Research Institute's Phosphate (NBRIP) growth medium: Glucose 10 g, ammonium sulfate (NH_4)₂SO₄ 0.1 g, potassium chloride KCl 0.1 g, magnesium sulfate $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.25 g, magnesium chloride $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ 0.25 g, phytic acid sodium salt 5.0 g, agar 15.0 g [6].
3. Minimal medium: Glucose 100 mM, Tris-Cl (pH 8.0) 100 mM, potassium nitrate (KNO_3) 50 mM, Sodium chloride NaCl 8.9 mM, magnesium sulfate ($\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$) 2 mM, calcium chloride (CaCl_2) 100 mM, Na-phytate 0.3% (filter sterilized, add separately before pouring of plates), agar 2.5%, methyl red (pH indicator) 0.03% [7].

2.2 Enzyme Activity

2.2.1 Acid Phytase and Alkaline Phytase Activity [7]

1. Media for growth: Maltose 10 g, peptone 10 g, beef extract 5 g, magnesium sulfate $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 1.0 g, pH 7.0.
2. NaCl 0.85%.
3. Na-acetate pH 4.5 (for acid phytase).
4. 0.1 M of Tris-Cl pH 8.0 (for alkaline phytase).
5. Sonicator.

2.2.2 Acid Phosphatase and Alkaline Phosphatase Activity [7]

1. Na-acetate buffer (pH 4.5) 0.5 M.
2. Tris-Cl (pH 8.0) 0.5 M.
3. *p*-nitro phenyl phosphate (pNPP) 0.5 M.
4. Magnesium chloride (MgCl_2) 0.1 M.
5. NaOH-2N.

2.2.3

*Alkaline Phosphatase**Activity [8]*

1. Modified Universal Buffer (MUB) pH 6.5: Tris-hydroxymethylaminomethane 2.42 g, maleic acid 2.3 g, citric acid 2.8 g, boric acid 1.26 g. Dissolve it in 800 ml of milliQ water. Adjust the pH at 6.5 with 10 M with sodium hydroxide.
2. *p*-Nitrophenyl Phosphate 0.115 M: Dissolve 1.927 g of di-sodium-Nitrophenyl Phosphate tetrahydrate in MUB. Dilute the solution to 50 ml and store it in refrigerator.
3. Calcium chloride 0.5 M: Dissolve 73.5 g of $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ in water and dilute the solution to 1:1.
4. Sodium hydroxide 0.5 M: Dissolve 20 g of NaOH in water and dilute the solution to 1:1.
5. Standard *p*-Nitrophenyl Phosphate solution: Dissolve 1.0 g of *p*-Nitrophenyl in water and dilute the solution to 1:1. Store the solution in refrigerator.

2.2.4 Estimation

*of Phosphate by**Vanadomolybdate Method**[9]*

1. Solution I: Ammonium molybdate ($(\text{NH}_4)_6 \cdot \text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$) 10.0 g, dissolve it in 200 ml of warm distilled water.
2. Solution II: Ammonium metavanadate ($(\text{NH}_4)_2 \text{VO}_3$) 0.5 g, dissolve it in 200 ml of warm distilled water.
3. Solution III: Mix Solution I and Solution II and add 125 ml of concentrated HNO_3 (Solution can be stored at 4 °C for an indefinite period).

3 Methods

3.1 Isolation***of Phytin Mineralizing******Microorganisms***

1. Dilute the soil by serial dilution method (up to 10^{-1} – 10^{-5}) with 0.85% sterile NaCl solution.
2. Take 1 ml of suspension from individual samples and spread it on either of selective media. Incubate the plates for 24 h at 37 °C and 120 h at 30 °C for isolation of phytin mineralizing bacteria and fungus, respectively. Monitor the plates daily.
3. On phytin mineralizing (PM) media and National Botanical Research Institute's Phosphate (NBRIP) growth medium, the organisms which form halo zone around the colony indicate mineralization of phytin. Phytin mineralizing microorganisms show pink colored (pH ~ 5 or below) zone on yellow colored (pH 8.0) plates on minimal media with Na-phytate as a sole source of phytin.
4. Isolate the colony and purify it by gradual subculturing. Determine the solubilization index by using following formula [10].
Solubilization Index (SI) = (Halo zone + Colony diameter)/Colony diameter

3.2 Enzyme Assay

3.2.1 Estimation of Acid Phytase and Alkaline Phytase Activity

1. Grow isolated phytin mineralizing microorganisms in 100 ml of growth media.
2. Use culture supernatant for determination of extracellular enzyme activity.
3. To determine cell-associated enzyme activity, wash the pellets for three times with 0.85% NaCl. Resuspend the washed pellets into 5 ml of 0.85% NaCl. Sonicate the cell suspension for 3.33 min at 9.9 s on/off pulse with 28% magnitude using ultrasonicator. Sediment the pellet by centrifugation and use supernatant for assay.
4. Take 0.7 ml of Na-acetate buffer pH 4.5 (for acid phytase) or 0.1 M of Tris-Cl buffer pH 8.0 (for alkaline phytase) with 0.7 mM of Na-phytate.
5. Make final volume up to 1 ml with either sample enzyme solution or water (blank) and incubate it at 37 °C for 30 min.
6. Estimate the amount of released Pi with ammonium molybdate method [9]. (.....).
7. Calculate the phytase activity. One phytase unit is defined as the amount of enzyme releasing $1\mu\text{mol P min}^{-1}$. Specific phytase activity is defined as mU mg^{-1} protein.

3.2.2 Estimation of Acid and Alkaline Phosphatase Activity

1. Take 0.1 ml of either Na-acetate buffer (pH 4.5) (acid phosphatase) or Tris-Cl (pH 8.0) (alkaline phosphatase) buffer, 0.1 ml of pNPP, and 0.1 ml of 0.1 M magnesium chloride (MgCl_2) solution.
2. Add different aliquots of sample to the assay mixture and make final volume up to 2 ml with distilled water.
3. Incubate it in dark at 30 °C for 30 min.
4. Stop the reaction by addition of 2 ml NaOH.
5. Vortex the reaction mixture and estimate the released pNPP by taking its optical density at 405 nm.
6. Calculate the phosphatase activity. One unit of phosphatase activity is defined as the amount which releases $1\mu\text{mol of } p\text{-nitrophenolate min}^{-1}$.
Specific phosphatase activity is defined as mU mg^{-1} protein.

3.2.3 Estimation of Alkaline Phosphatase Activity [8]

1. Take 1 ml of filtered broth, add 4 ml of MUB, 0.25 ml of toluene, and 1 ml of pNPP solution in a flask and mix it thoroughly. Use water as a blank.
2. Stopper the flask and incubate it at 37 °C for 1 h.
3. Remove the stopper and add 1 ml of 0.5 M calcium chloride with 4 ml of 0.5 M sodium hydroxide. Swirl the flask and

observe the intensity of yellow color at 400 nm by spectrophotometer.

4. Calculate the content of p-Nitrophenol in the filtered broth by standards using different concentration. (Calibration graph can be prepared by diluting 1 ml of p-Nitrophenol in 100 ml of flask. Pipette out aliquots of 0, 1, 2, 3, 4, and 5 ml of standard solution. Adjust the volume up to 5 ml by water and proceed it as described above.)

3.2.4 Quantitative

Estimation of Phosphate by Vanadomolybdate Method

1. Take 1 ml of test solution into test tube. Add 4 ml of solution III into solution and check for the development of yellow color and record optical density at 420 nm against blank.
2. Use a solution of KH_2PO_4 as a standard.

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Isolation and Screening of Silicate Solubilizing Microbes: Modern Bioinputs for Crops

Chandrashekhar D. Patil, Bhavana V. Mohite, Rahul K. Suryawanshi, and Satish V. Patil

Abstract

Pertaining to the importance of silica as fertilizer for various crops, silica solubilizers become new component of biofertilizers for sustainable crop and fertility management. Importance of silica metal has been identified for tolerance of biotic and abiotic factors, growth enhancement, etc. Soil is rich source of silica however; it is majorly available in un-utilizable form of silicates. Therefore, silicate solubilizing microbes are essential part of micronutrient management program. The present protocol describes method of isolation and screening of silicate solubilizers from various sources. The application of pH indicator dyes in medium will also help to find out microbe-mediated mechanism of silicate solubilization. Medium with differential sources of immobilized or polymeric silicates will allow isolation of potent and versatile silicate solubilizers useful as modern bioinputs for crops.

Key words Silicate, Rice, Drought, Organic acid, Ammonia

1 Introduction

The silicon (Si) is one of the abundant component of soil. Si was previously considered as nonessential element for crop development, but recent findings proved significance of Si to induce tolerance of biotic and abiotic factors and growth enhancement by carbon photoassimilation in crops like rice, maize, wheat, etc. [1–3] (Fig. 1) and play vital role in development of pathogen resistance in silicon accumulating crops like rice. Various diseases of rice crop, rice blast, powdery mildew, brown spot, stem rot and sheath brown rot on rice were found to be successfully managed by new bioinputs such as silicon fertilizer application [5–7]. Additionally, Si is also known to facilitate the availability of other micro- and macronutrients such as phosphorus, potassium, zinc, and copper [4] (Fig. 1).

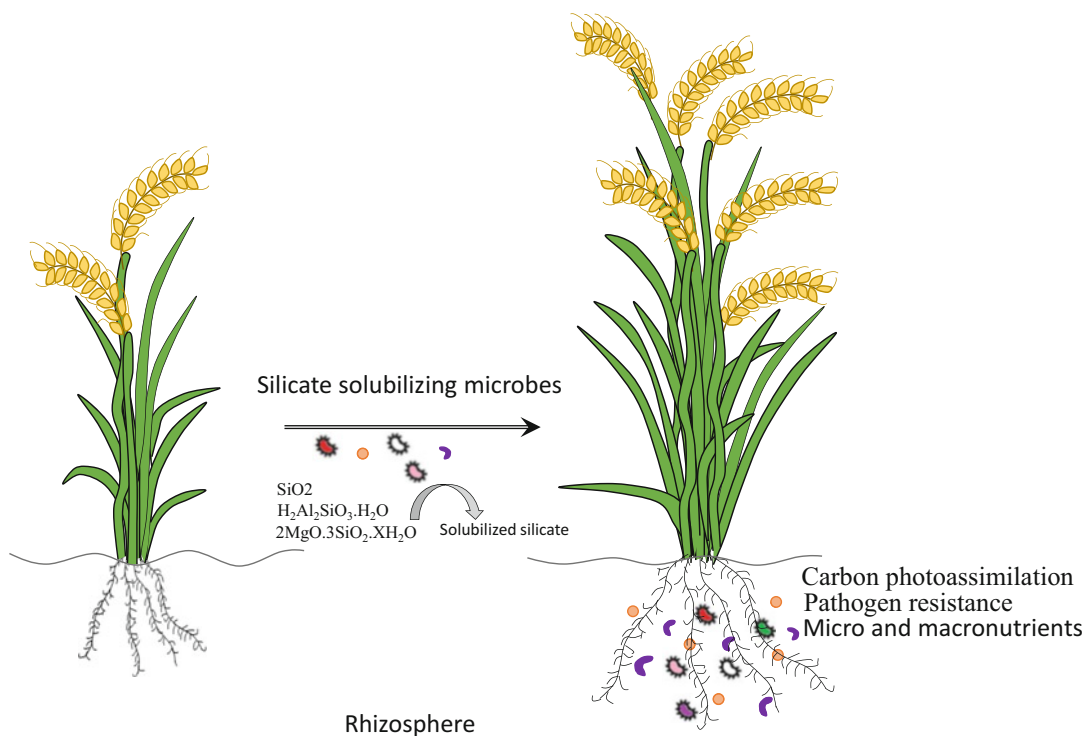


Fig. 1 Graphical representation of silicate solubilizing microorganisms for improving silicate bioavailability

Silicon is abundantly available in soil but in immobilized or mineral form which is unavailable for direct assimilation by plant. The need of plant is fulfilled by extraneous addition of silicon fertilizers, but at majority times silicon fulfillment is done by adding industrial slag, which causes other heavy metal contamination in farm soil. Besides these, silicon fertilizers in market such as bentonite, mica feldspars, and diatomaceous earth are present but they contain very little bioavailable Si and so require application of higher dose. Currently, potassium silicate is applied as popular silicon fertilizer but it is too expensive, and may cause phytotoxicity, eutrophication, etc. The application of Si solubilizing biofertilizers proved most ecofriendly, cheap, and significant to fulfill the crop Si need.

Silicon solubilization is first reported by Aleksandrov et al. [8] during their work on microbial phosphoric acid liberation by silicate bacteria. Various microbes were reported for silicon depolymerizing/solubilizing potential, i.e., *Rhizobium*, *Burkholderia*, *Pseudomonas* sp. [9]. Some studies proved that fungi actively solubilize the silicate mineral from rock mineral, e.g., *Fusarium oxysporum* [10, 11].

The naturally occurring silicon in the form of soil minerals is majorly converted by the process of withering into the monosilicic acid which is assimilated by plant roots. The withering of silicon

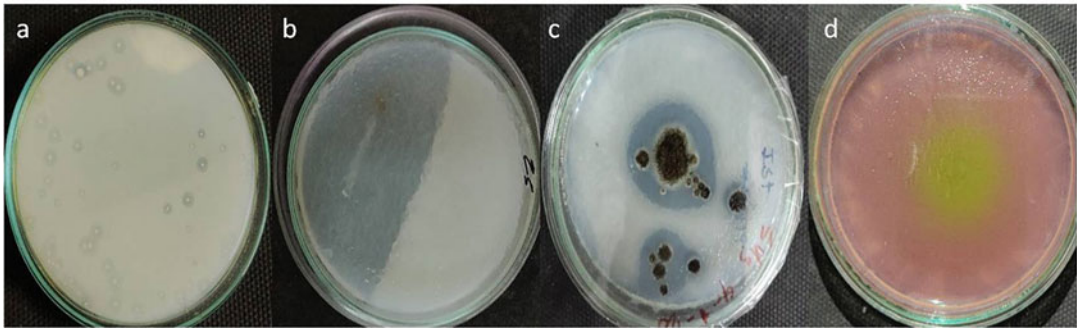


Fig. 2 (a) Isolation of silicate solubilizers on silicate agar, (b) silicate solubilization by bacteria, (c) silicate solubilization by fungi, (d) confirmation silicate solubilization mode of action by pH indicator silicate (Phenol red). (Isolates From Dr. SV Patil, Laboratory School of Life Sciences, KBC, NMU, Jalgaon)

minerals take place by various ways, i.e., by organic and inorganic acids, actions of divalent cations, by nucleophilic attacks of alkali compounds, etc. Majority of these products are produced by microbial action.

Recent studies revealed that the foliar applications of silicate solutions stimulate growth of wheat cereals, soybean, rapeseed, sugar beet, potato, meadows, berries and vegetables, as well as orchard and ornamental plants in diverse abiotic stress like drought by stimulating chlorophyll production, maintaining water content and cellular membrane integrity [12].

1.1 Principle of Assay

Plant rhizosphere is a rich source of silicate solubilizers. Screening of silicate solubilizers uses a classical approach of cultivating test microorganisms isolated from plant rhizosphere on complex silicate mixture containing minimal medium and isolating microbes capable of making zone of clearance (Fig. 2). pH indicator dye can be used to detect acid or alkali based microbial mechanisms of silicate solubilization. The solubilized zinc and its presence in plant can further be estimated through colorimetric, coupled plasma mass spectrometry or absorption/emission spectrometry.

2 Materials

1. Rhizospheric soil sample from the agricultural crops such as rice, wheat or maize and root nodules of the bean crops (*see Note 2*).
2. Sterile saline tubes (10 tubes) for serial dilution of the soil samples.
3. **Following media will be needed for isolations of different silicate solubilizers**
 - (a) **Sterile Silicate Agar for Bacteria:** (g/L) Magnesium sulfate 0.1, Calcium carbonate 0.1, magnesium trisilicate [$\text{Mg}_2\text{O}_8\text{Si}_3$] 1.0 or Potassium alumino silicate 1.5,

- Glucose 1.0, Ferric chloride 0.005, Calcium phosphate 1.0, ammonium sulfate 0.2, Agar 30 g, Final pH (at 25 °C) 7.2 ± 0.2 + 5 ml of 10 mg% Phenol red or 20 mg% bromothymol blue per liter (*see Note 1*).
- (b) **Sterile Silicate Agar for Fungi:** (g/L) Glucose 0.5, Yeast extract 0.5, Ammonium sulfate 0.4, magnesium trisilicate [$\text{Mg}_2\text{O}_8\text{Si}_3$], Potassium aluminosilicate 1.5, Calcium phosphate 1.0, Agar 30.0, Final pH (at 25 °C) 6.5 ± 0.2 + 5 ml of 10 mg% Phenol red or 20 mg% bromothymol blue per liter (*see Note 1*).
- (c) **Sterile Silicate Agar for Actinomycetes:** (g/L) Magnesium sulfate 0.1, calcium carbonate 0.1, potassium aluminosilicate 1.5, Starch 2 g ferric chloride, asparagine 0.01, Casein 0.02 g, calcium phosphate 1.0 g, ammonium sulfate 0.2, Agar 30 g, final pH (at 25 °C) 7.2 ± 0.2 + 5 ml of 10 mg% Phenol red or 20 mg% bromothymol blue per liter (*see Note 1*). (d) Aleksandrov medium used for potassium solubilizers is also applicable for isolation of silicate solubilizers.

3 Methods

1. Take 1 g of collected soil sample, add in 9 ml of sterile saline, mix well by shaking and then serially dilute up to tenfold, i.e., 10^{-9} .
2. Prepare silicate agar for bacteria, fungi, and actinomycetes by using pure distilled water in clean 250 ml Erlenmeyer flasks and sterilize them at 121 °C for 20 min under 15 psi pressure.
3. Let the media to cool at 40 °C, and mix well the settled silicate and carbonate.
4. Pour it into the sterile petri plates and allow solidifying at room temperature for 2–3 h. Also prepare control media plates of each culture media without silicate.
5. Take loopful of soil suspension from 10^{-5} to 10^{-6} dilution tube and streak on selenite containing silicate agar plates.
6. Incubate the plates at room temperature approximately up to 48 h; observe the plates for growth and zone of clearance around colony (Fig. 2).
7. Compare the silicate containing plates with the control plates to examine the solubilization of Si and thereby formation of zone around the colonies.
8. These isolates can be further purified in liquid enrichment medium supplemented with magnesium silicate at low concentration of silicate. Isolate specific media required for the

conversion of silicate into depolymerized form of soluble silicon/nanoparticles can be further optimized and the silicon particles can be characterized for solubilization properties (Fig. 2).

9. For confirmation of solubilizing mechanism, use pH indicator in the medium. Add 3–5 ml of 10 mg% phenol red or 10 mg% bromophenol blue and mix well (*see* **Note 3**).
10. The color change will indicate whether solubilization might be due to acid or alkali.
11. **Estimation of Solubilized silica:** Centrifuge the broth medium of silica solubilizers, take 5 ml of supernatant, mix with 15 ml 2.5% boric acid and 5 ml 54% ammonium molybdate, incubate for 5 min and add 1 ml of 0.5% ascorbic acid solution, mix the solution by vortex and measure absorbance at 650 nm. The concentration of silicate could be determined by the standard graph of SiO_2 by spectrophotometric method.
12. **Bioassay and analysis of Si content in test plant:** Plant ten rice *oryza sativa*/*Triticum aestivum*, sugar cane, maize, wheat seedling in the 5×3 cm pots, filled with sterilized black cotton soil and one pot can be inoculated with silicate solubilizers inoculum. The uninoculated pots should be considered as control which should be allowed to grow for 3 weeks, then uproot the plants, wash with distilled water and dry it at room temperature and measure weight of control and test plant. Dry the plant at 60°C for 3 h and use for silicate analysis in plant (*see* **Notes 4** and **5**).
13. **Analysis of Si content of the plant:** Ground the 0.5–1.0 g of dried biomass of control and test plant. As per method described by Kaya et al [13] take 0.5 g of the lyophilized crushed powder and soak in 0.5 M HCl for 1 h, then wash by distilled water and dry in oven. Add the dried powder in the mixture of nitric acid, sulfuric acid, and perchloric acid (10:1:4 v/v/v) keep it for 1 h, then this digested sample can be analyzed by coupled plasma mass spectrometry (*see* **Notes 4** and **5**).

4 Notes

1. For isolation of silicate solubilizers, various polymeric silica sources can be used in medium such as quartz, bentonite, magnesium trisilicate), aluminum silicate, calcium fuller's earth, kaolin potassium alumino silicate), potassium, aluminum and calcium silicates, phyllosilicates, etc.
2. For the rhizospheric endophytes and the rhizobia, surface sterilize the material with 70% ethanol or 0.01% mercuric chloride. Then crush the sample in sterile saline, further dilute and

streak the specific dilution on sterile silicate solubilization agar medium.

3. Add pH indicators like bromocresol green, phenol red, bromophenol blue, etc. in medium to know mechanism of solubilization of silicates. Prepare pH indicator solution in 70% ethanol to avoid contaminations. pH indicators can be added after sterilization.
4. Many times these depolymerized or solubilized Si particles are in nanoform as hence as nano silicates, which have various significant potential for various industrial and agriculture applications. The nanoforms are characterized by various spectroscopic methods. Si-nanoparticle is characterized by UV–Vis absorption spectrophotometer by recording the spectra (200–500 nm).
5. The lyophilized powder is used for further particle characterization such as particle size, shape, and crystallinity by using scanning electron microscopy, transmission electron microscopy, dynamic light scattering (DLS), and X-ray diffraction.

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Chapter 31

Isolation of Selenium Biotransforming Microbes as New Age Bioinputs

Pradnya B. Nikam, Narendra Salunkhe, Vishal Marathe, Bhavana V. Mohite, Satish V. Patil, and Vikas S. Patil

Abstract

Selenium (Se) has a very narrow gap between its toxicity and benefits to the different forms of life which makes it as an essential micronutrient for living creatures including its importance in plant growth. It is highly soluble having toxic form in the environment such as the oxyanions; selenate and selenite needed to be transformed to the less toxic elemental selenium which has wide number of benefits to the ecosystem, if provided in appropriate required amount. Till now most of the microorganisms have been studied for biotransformation of selenite by using them as electron acceptor in the respiratory mechanism and ultimately reduced to elemental selenium in the form of nanoparticles. Despite of this, many of the microbes are unexplored and the Se-nanoparticles still less studied for their applications. This chapter explains a simple method for isolating the selenium biotransforming microorganisms from nearby soil sample into the red elemental Se in its nano form.

Key words Selenium, Selenite, Thioredoxin reductase, Selenobacter, Antioxidant

1 Introduction

Selenium (Se) has been proven as an essential micronutrient for almost all living organisms, which is also found important for plants in various aspects. Its presence in the environment ranges from inorganic oxyanions to organic selenium containing amino acids. In human beings, various enzymes such as glutathione peroxidase, thioredoxin reductase, iodothyronine deiodinase, and formate dehydrogenase have selenium as their important component and thus function as an antioxidant, preventing tumors, metabolism, other therapeutic uses and even in reproduction [1, 2]. In the environment, Se exists as highly soluble oxyanions such as Selenate (SeO_4^{-2}) and Selenite (SeO_3^{-2}) which are toxic for humans, hence

Satish V. Patil and Vikas S. Patil contributed equally to this work.

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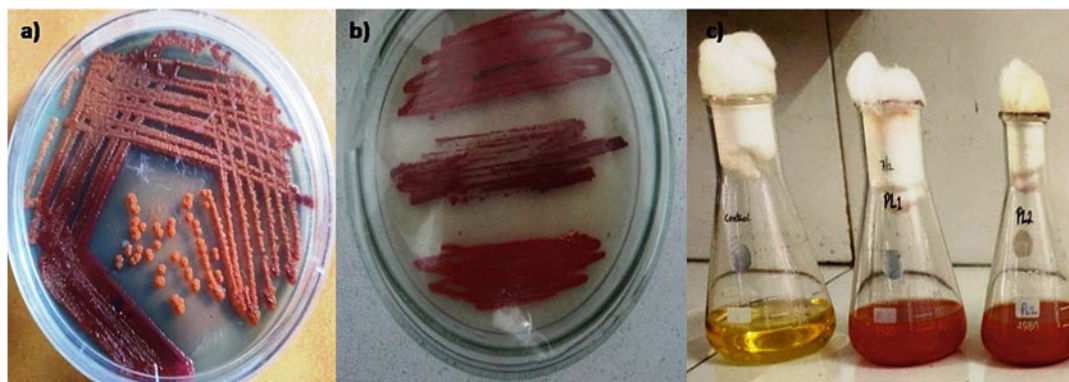


Fig. 1 (a and b) Isolated colonies of selenium transforming bacteria showing brick red color colonies; (c) Transformation of selenium in enrichment medium showing color change from yellow (control) to brick red (test isolates, PI1 and PI2) (Source: Satish V Patil's Laboratory KBC NMU, Jalgaon)

transformed into less toxic and less soluble elemental form (Se^0) by different microbial species through Se cycle. Many of the plants are seen to accumulate these Se oxyanions from the soil and form selenocysteine or may carry formation of selenomethionine, further converting it into volatile dimethylselenide (DMSe). Though Se is not essential for plant growth, it helps the crops to withstand the drought conditions, protects against oxidative stress and sometimes from the pests too [3].

As the distribution of selenium is uneven in the environment, it becomes important to provide it as less toxic and less soluble Se^0 , mostly in colloidal form for the living creatures. This can be achieved by isolating the microbes from different areas especially the agricultural rhizospheric soil or also from the endophytic microbes within the roots, as plants have the potential of accumulating the Se at any available concentrations from the soil. The agricultural rhizospheric soil and nodule samples are preferred considering the compatibility of synthesized Se-nanoparticles for further applications in agricultural practices and also most of them will be non-pathogenic [3–5]. When the sample is enriched in the growth medium supplied with selenite or selenate as a Se source, within 24–48 h the visual brick red color starts appearing along with the growth which signifies the transformation of Se by the respective organism (Fig. 1). This transformation relies on the growth phase and also the initial Se concentration in the medium, as reported earlier. Several reports declare this transformation as synthesis of the Se^0 nanomaterial either extra- or intracellular by the microbes [6–8].

2 Materials

All the culture media used are from HiMedia (Mumbai, India) including the sodium selenite. Pure grade distilled water was used for dilution.

1. Rhizospheric soil sample from the agricultural crops such as wheat or maize and root nodules of the bean crops.
2. Sterile nutrient agar media for bacterial isolation.
3. Sterile Sabouraud dextrose agar for fungal isolation.
4. Sterile yeast extract mannitol agar (YEMA) to culture root nodule bacteria (*Rhizobia*).
5. Sterile saline tubes (10 tubes) for serial dilution of the soil samples.
6. Sodium selenite (Na_2SeO_3) (MW—172.94 g/mol).

3 Methods

1. Take 1.0 g of collected soil sample, add in 9.0 ml sterile saline, mix well by shaking and then further dilute up to tenfolds, i.e., 10^{-9} (*See Note 2*).
2. Prepare nutrient agar media, Sabouraud dextrose agar, and yeast extract mannitol agar by using pure distilled water in clean 250 ml Erlenmeyer flasks and sterilize them at 121 °C for 20 min under 15 psi pressure in the autoclave.
3. Let the media cool at 40 °C, and then add sodium selenite in nutrient agar media as well as in Sabouraud dextrose agar (5 mM per volume of medium). Rotate flask clockwise and anticlockwise for uniform distribution of selenite (*See Note 1*).
4. Pour it into the sterile petri plates and allow solidifying at room temperature for 2–3 h. Also prepare control media plates of each culture media without sodium selenite.
5. Take loopful of soil suspension from 10^{-5} to 10^{-6} dilution tube and streak on selenite containing nutrient agar media and Sabouraud dextrose agar plates.
6. Incubate the plates at room temperature up to approximately 48 h; observe the plates for growth changed to red color.
7. Compare the sodium selenite containing plates with the control plates to examine the exact biotransformation of Se and formation of red colored colonies.
8. These isolates can further be inoculated in liquid enrichment broth medium supplemented with sodium selenite at 5 mM

concentration for selenite biotransformation and conversion to the elemental Se-nanoparticles which can be optimized and characterized further.

9. The synthesized nanoparticles are separated by centrifugation at 10,000 rpm for 5–8 min, and collecting the pellet and purifying with double distilled water and ethanol by washing three times to remove the untreated sodium selenite or other impurities. The nanosuspensions then pass through the dialysis membrane (6000–8000 KD cutoffs). The purified particles then lyophilized and stored at low temperatures which will be used for characterization by various spectroscopic methods.
10. Se-nanoparticle is characterized by UV–Vis absorption spectrophotometer by recording the spectra (200–500 nm).
11. The lyophilized powder is used for further particle characterization such as particle size, shape, and crystallinity by using scanning electron microscopy, transmission electron microscopy, dynamic light scattering (DLS), and X-ray diffraction.

4 Notes

1. Avoid adding sodium selenite into the media preparation before autoclaving as this may result into precipitation of the salt and forming red color due to heat and pressure, prior to the addition of microbial culture sample.
2. For the rhizospheric endophytes and the rhizobia, surface sterilize the material with 70% ethanol or 0.01% mercuric chloride washing. Then crush the sample in sterile saline, further dilute and streak the specific dilution on sterile YEMA or endophyte isolation agar medium.

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Part III

Estimation of Phytohormones by Beneficial Microbes



Chapter 32

Auxin

Dixita Panchal, Jemisha Mistry, and Dhruti Amin

Abstract

Auxin is commonly known as phytohormone and it regulates the developmental mechanism of plants. Generally, auxin is produced by plants and some microorganisms. Microbial synthesis of the phytohormone auxin has been known for long years. Auxin biosynthesis in bacteria can occur via multiple pathways as has been observed in plants. Indole-3-acetic acid (IAA) is most abundant, naturally occurring auxin and plays a major role in plant growth and protects the plant from various biotic and abiotic stress. This chapter gives rapid and efficient protocols for screening for auxin producing microbes, colorimetric and chromatographic methods for detection of auxin.

Key words Phytohormone, IAA, Auxin producing microbes, Qualitative and quantitative analysis

1 Introduction

An auxin is a phytohormone derived from amino acid L-Tryptophan (TRP) known to regulate plant growth and development. Auxin is produced by plants and several microorganisms, especially rhizosphere bacteria. It is even assumed that over 80% of the bacteria isolated from the rhizosphere are capable to synthesize IAA [1, 2]. Indole-3-acetic acid (IAA) is the most common physiological active auxin. Tryptophan is a key precursor for the synthesis of IAA. Mostly it is observed that addition of tryptophan to culture media resulted into higher IAA production. Synthesis of IAA occurs by two different pathways: (1) TRP is converted to IAA by deamination to indole-3-pyruvic acid followed by decarboxylation to indole-3-acetaldehyde which further oxidized to IAA and (2) TRP is decarboxylated to indole-3-acetamide which is hydrolyzed to IAA [3, 4]. Detection of IAA produced by plants and microorganisms is determined by using colorimetric and chromatographic methods [5–7]. IAA producing ability of microorganisms is useful for identification and also serves as a valuable indicator of physiological roles and ecological significance of these microorganisms in the environment [5].

2 Materials

2.1 Screening for Auxin Producing Bacteria [8]

1. Bacterial isolates.
2. Nutrient broth (10 peptone, 10 g beef extract, 5 g sodium chloride pH 7.3 ± 0.1 per liter).
3. Orthophosphoric acid.
4. Salkowski reagent (50 ml of 35% perchloric acid and 1 ml of 0.5 M FeCl_3).
5. Centrifuge tube.
6. Centrifuge.

2.2 Estimation of Auxin by Spectrophotometer/Microplate Method [5, 9]

1. Bacterial isolates.
2. Tryptic soy agar (15 g pancreatic digest of casein, 5 g peptic digest of soybean meal, 5 g NaCl, 15 g Agar, pH 7.3 ± 0.2) for 1 l.
3. Growth medium (5 g glucose, 0.025 g yeast extract, 0.204 g L-tryptophan) for 1 l.
4. Salkowski reagent.
5. UV-VIS spectrophotometer.

2.3 Determination of Auxin by Thin-Layer Chromatography [7]

1. Ethyl acetate.
2. Flash evaporator.
3. Methanol.
4. Whatman LK6DF silica gel thin-layer chromatography plates.
5. CEF (Chloroform: ethyl acetate: formic acid, 50:40:10).
6. EIA (Ethyl acetate: isopropanol: ammonia, 45:35:20).
7. Ehrlich's reagent (0.2% *p*-dimethylaminobenzaldehyde in 1:1 mixture of 95% ethanol and concentrated HCL).
8. Standard IAA.

2.4 Determination of Auxin by HPLC [7]

2.4.1 Method I

1. Injection solution (Sodium citrate 500 $\mu\text{g}/\text{ml}$, EDTA 10 mM, Methanol 20%, butylated hydroxytoluene 2 $\mu\text{g}/\text{ml}$).
2. 0.05 M phosphate buffer (pH 3) containing 18% methanol v/v.
3. Waters Associates M6000A solvent delivery system.
4. Waters Associates WISP 710B autosampler.
5. BAS electrochemical LC-3 detector.
6. Kratos FS950 fluorescence detector.

2.4.2 Method II

1. Dupont ODS reserved-phase column.
2. 25% tetrahydrofuran (pH 3.0 adjust with phosphoric acid).
3. Waters Associates M6000A solvent delivery system.
4. Rheodyne 7125 injector.
5. Amino filter fluorometer.

3 Methods

3.1 Screening of Auxin Producing Isolates [8]

1. Inoculate 24 h growth culture of test isolates in nutrient broth with tryptophan.
2. Incubate culture tubes at 28 ± 2 °C for 3 days.
3. Use uninoculated growth medium as a control.
4. Centrifuge 3 ml of bacterial cultures at 6000 rpm for 30 min.
5. 2 ml of supernatant mix with 2 drops of orthophosphoric acid and 4 ml of Salkowski reagent.
6. Allow mixtures to stand for 25 min at room temperature in dark condition for color production.
7. Colonies showing color change from yellow to pink or red are auxin producing isolates.
8. Store the purified strain as a glycerol stock at -80 °C for the further study.

3.2 Estimation of Auxin by Spectrophotometer or Microplate Method

Bacterial isolates showing auxin production are further used for quantitative analysis by spectrophotometry/microplate method.

3.2.1 Method I [5, 9]

1. Add 2 ml of culture suspension into 28 ml of growth medium and use sterile water as control.
2. Tubes to be capped, vortex and incubate in dark at 27 °C for 72 h.
3. Prior analysis for auxins, individual cultures are adjusted to 10^8 cells/ml at 500 nm absorbance with sterile water and filter through 0.2 μ m membranes.
4. Mix 3 ml of filtrate with 2 ml of Salkowski reagent and kept at dark for 30 min at room temperature.
5. Absorbance of color change is measured by using UV-VIS spectrophotometer at 530 nm.
6. To measure auxin concentration using a standard graph of indole acetic acid (IAA stock solution range 10–100 μ g/ml) (See **Note 1**).

3.2.2 Method II Microplate

Microplate method is a modification of the standard method [9].

1. Dispense 150 μ l of culture filtrate into well of 96-well microplate and add 100 μ l of Salkowski reagent.
2. Allow to react for 30 min.
3. Measure color intensity at 530 nm on a microplate reader.

3.3 Determination of Auxin by Thin-Layer Chromatography [7]

Procedure for production of auxin by bacterial isolates as shown in Subheading 3.1 steps 1 and 2.

1. Adjust culture supernatants to pH 3.0 and add ethyl acetate equal volume to culture filtrate.
2. Allow ethyl acetate to evaporate from a mixture using a flash evaporator and then solubilize extract in a minimal volume of methanol.
3. The methanol suspension spot on Whatman LK6DF silica gel thin-layer chromatography plates.
4. Plates are developed in two different solvent system CEF or ELA.
5. Spray Ehrlich's reagent on plate immediately after removal from developing chamber.
6. Detection of IAA by blue band with the R_f corresponding to that of IAA standards.
7. To determine IAA recovery of IAA by incorporating freshly prepared IAA standard in the culture medium before initiating the extraction procedure (Limit of detection of IAA standard is 0.10 μ g/ml).

3.4 Determination of Auxin by HPLC [7]

1. Dilute cultures supernatants into an injection solution before HPLC analysis.
2. Hydrolyze base conjugate of IAA by adjusting culture filtrate to pH 11.0, incubate at 60 °C for 60 min.
3. Then readjust the culture pH to 3.0 before dilution into the injection solution.
4. IAA analysis is done by two different methods are as follows.

3.4.1 Method I

1. Use HPLC system comprising reverse-phase C8 column (25 cm $\times$ 4.2 mm) and a mobile phase of phosphate buffer.
2. This system equips with BAS LC-22 controller and LC-23 column heater set at 50 °C.
3. Waters Associates M6000A solvent delivery system and Waters Associates WISP 710B autosampler set to inject 100 μ l samples.
4. The system should equip with two separate selective detectors connected in series.

5. The first detector, a BAS electrochemical LC-3 has working potential of 0.85 on glassy carbon vs. Ag/AgCl.
6. The second, a Kratos FS950 fluorescence detector, operate with medium pressure mercury lamp, a 280 nm interference filter, and emission filter with transmittance at 320 nm.
7. Calculate the ratio of peak heights from the fluorescence (F) and the electrochemical (EC) detectors for IAA standards.
8. For each sample, measure peak height corresponding to retention times of IAA obtained from chromatographs of each detector, and calculate F/EC ratio.

3.4.2 Method II

1. Use a Dupont ODS reserved-phase column of 7-nm particle diameter (4.6 mm × 25 cm) run at ambient temperature.
2. The mobile phase is 25% tetrahydrofuran.
3. Use Waters Associates M6000A solvent delivery system and Rheodyne 7125 as injector and inject 50 µl sample into it.
4. The system is equipped with an amino filter fluorometer (emitting at 320 nm and exciting at 280 nm).

4 Note

1. Salkowski's reagent reacts with IAA and does not interact with L-tryptophan, therefore this reagent is important for qualitative and semi-qualitative determination for the presence of the auxin hormone.

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Chapter 33

Abscisic Acid

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Abstract

Abscisic acid (ABA) is a phytohormone involved in a wide range of the plant's physiological processes. ABA acts as a stress indicator, either abiotic or biotic, in the plant. It plays a role in abscission, stomatal closure, fruit ripening, seed and bud dormancy, and senescence. This chapter provides an insight into a simple, rapid and reliable protocol for qualitative and quantitative analysis of the abscisic acid content from plant extracts by various chromatographic techniques.

Key words Stress-related hormone, Extraction, High-performance liquid chromatography, Gas-liquid chromatography-mass spectrometry

1 Introduction

The phytohormone abscisic acid (ABA) is involved in an extensive range of physiological processes of plants. ABA is known to mediate stomatal closure and water stress avoidance. In many plant species, the hormone level is directly related to plant capacity to tolerate water stress level in response to a decrease of leaf water potential or turgor [1, 2]. The ABA level in plants gets elevated if other stress factors like salinity, nutrient deficiency, or waterlogging arise and act as a stress indicator in plants. The extraction and purification process involves typically the partition of plant extracts against organic solvents [3], followed by chromatography (paper, thin-layer chromatography, high-performance liquid chromatography, or gas-liquid chromatography-mass spectrometry) [4]. The extraction procedures are time-consuming, and each extraction step generally results in some ABA loss. The low concentration of this hormone present in plant tissues makes its determination difficult. Gas-liquid chromatography or HPLC is the most commonly used method for the quantitative determination of ABA. An abscisic acid derivative formed by reaction with pentafluorobenzyl bromide allows sensitive detection by gas-liquid chromatography electron

capture detection. Compared to the methyl ester derivative, the pentafluorobenzyl product of abscisic acid was four times more sensitive to electron capture detection. It is stable at room temperature in the presence of ultraviolet light, and derivatization is rapid. The molecular weight of the new compound can determine by using gas-liquid chromatography-mass spectrometry [5].

2 Materials

2.1 Elution of ABA from TLC Followed by Gas-Liquid Chromatography [6–8]

2.1.1 Thin-Layer Chromatography

1. Fresh leaves from plant grown under stress condition.
2. Abscisic acid standard.
3. Acetone.
4. Water.
5. Ethyl acetate.
6. Sonicator.
7. TLC assembly.
8. UV light.

2.1.2 Gas-Liquid Chromatography

1. Nitrosoethylurea (*see Note 1*).
2. Nitrosomethylurea (*see Note 1*).
3. Petroleum ether.
4. Cyclohexane.
5. Gas-liquid chromatography instrument with electron capture detector.

2.2 Gas-Liquid Chromatography-Mass Spectrometry [5, 9]

1. ABA sample (*see Note 2*).
2. GLC-MS equipment.

2.3 High-Performance Liquid Chromatography [9]

1. Soybean ABA extract.
2. [^{14}C] ABA.
3. HPLC column (μ Bondapak C18, μ Porasil, and μ Bondapak-NH₂) (Waters Associates).
4. HPLC instrument.

3 Methods

3.1 Elution of ABA from TLC Followed by Gas-Liquid Chromatography

3.1.1 Thin-Layer Chromatography

1. Collect leaves from a different plant growing under with or without stress condition.
2. Cut it into 2–3 mm pieces and weigh 300 mg.
3. Prepare extract by ultrasonic disintegration in acetone: water (9:1) extraction solvent mixture to fresh weight ratio of 10:1 in the dark at 0 °C for 5 min setting 3 (50 W).
4. Keep extract in a refrigerator for 1 or 2 days.
5. Use the supernatant as a sample for thin-layer chromatography.
6. Load 300 µl of extract and ABA standard separately on TLC plates precoated with silica gel GF₂₅₄ and use ethyl acetate as mobile solvent.
7. Mark ABA band using UV light.
8. Remove the band of silica gel containing the ABA from each plate.
9. Elute in 2 × 175 µl of water-saturated distilled ethyl acetate using suction.

3.1.2 Gas-Liquid Chromatography

10. Prepare ethyl abscisate from ABA and diazoethane (diazoethane preparation: obtain petroleum ether solution at 40–60 °C from nitrosoethylurea and 40% KOH solution).
11. Add 50 or 100 µl of 2×10^{-7} M of EtABA (2.92 or 5.84 ng) in cyclohexane to the elute sample as an internal standard.
12. Esterify the ABA with diazomethane (diazomethane preparation: distilled petroleum ether at 40–60 °C using nitrosomethylurea and 40% KOH).
13. Add 50 µl of a weak diazomethane solution to the ABA solution.
14. Keep the mixture in the dark approx. 5 min until the reaction is complete.
15. Evaporate the solvent and dissolve the residue in cyclohexane.
16. Inject 100 µl and 2 µl of this solution into the gas-liquid chromatograph equipped with electron capture detector.
17. Use the ratios of the areas for MEABA and ETABA to calculate the ABA content (nanograms per gram fresh weight of leaves).

3.2 Gas-Liquid Chromatography-Mass Spectrometry (GC-MS)

1. Further characterization of ABA extract can be possible by using GC-mass spectrometry.
2. Record mass spectra of ABA extracts by a Finnigan model 4021 GLC-MS equipped with a 1.85 m × 2 mm i.d., 3% OV-17 glass

column and set program between 200 and 250 °C at 5 °C per min.

3. Use helium as a carrier gas at a flow rate of 25 ml/min.
4. Perform ionization at 70 eV with a source temperature of 200 °C.
5. Keep scan rate at 0.95 scans per second.

3.3 High-Performance Liquid Chromatography

1. HPLC procedure is for the quantification of ABA (*see Note 3*).
2. Require commercially available HPLC columns packed with μ Bondapak C18, μ Porasil, and μ Bondapak-NH₂ (Waters Associates) 30 cm $\times$ 4 mm i.d. with a particle size of 10 μ m for ABA analysis.
3. Prepare extract from 15.05 g of soybean seeds as per standard method [9].
4. Divide the extract into separate subsamples for triplicate analysis through the chromatographic systems of μ Bondapak C18, μ Porasil, and μ Bondapak-NH₂.
5. Take an internal standard of 44 μ g of [14C] ABA and add to the fraction from the preparative HPLC procedure (containing approximately 160 μ g of endogenous ABA) before subdivision.
6. Collect the solvent elution zone containing the ABA peak from the μ Bondapak-NH₂ and μ Porasil columns.
7. Quantify the ABA in the respective subsamples based on peak height compared to ABA standards.

4 Notes

1. For detailed preparation of nitrosoethylurea and nitrosomethylurea, refer to [8].
2. For gas-liquid chromatography-mass spectrometry (GC-MS): Parameter (such column size, temperature) may vary as per instrument type.
3. For the preparation of plant extract for HPLC analysis, refer to [9].

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Cytokinins

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Abstract

Cytokinins are a group of plant growth regulators that promote cell division and differentiation of plant root and shoots. It also affects apical dominance, axillary bud growth, and leaf senescence of plant. The development of sensitive analytical methods for the determination of cytokinin levels in plant tissues is essential for elucidating the roles of cytokinins in life science. The analytical methods for cytokinins are designed to meet the requirements for cytokinin analysis in complex matrices using High-Performance Liquid Chromatography (HPLC) and Tandem Mass Spectrometry (MS/MS).

Key words Cytokinin, Phytohormones, HPLC, MS/MS

1 Introduction

Plant hormones are naturally occurring secondary metabolites that play key roles in the whole life cycle of plants. Cytokinins are a group of plant hormones [1, 2]. Certain cytokinins, kinetin and zeatin, show significant anti-aging, anti-carcinogenic, and anti-thrombotic effects [3].

Frequently these molecules act at low concentrations and play key roles in ecological interactions between plants and other organisms [4]. Therefore, quantification of phytohormones is an essential step to understand their functions in plant metabolism and ecological interactions. Cytokinins influence various traits of plant growth, development, and physiology such as seed germination, apical dominance, flower and fruit development, leaf senescence, and plant–pathogen-interactions. Therefore, rapid analysis of cytokinins is of great importance to plant physiologists and scientists from other disciplines, especially, in view of their potential role in suppressing mammalian tumor growth [5]. Plant tissue extracts contain complex multicomponent mixtures and endogenous cytokinins concentrations are very low (less than 50 pmol/g of fresh weight). This makes exhaustive analysis of cytokinins content

difficult. Sensitive and selective analytical tools are required for the determination of endogenous cytokinins levels in plant tissues [6]. Quantitative analysis of plant hormones is increasingly important for in-depth study of their biosynthesis, transport, metabolism, and molecular regulatory mechanisms [7]. For biological samples, HPLC is still the major tool in analyses and purification of cytokinins [8].

2 Materials

2.1 HPLC

1. Ammonium Acetate.
2. Diethylaminoethyl (DEAE).
3. C18 Sep-Pak cartridge.
4. Waters HPLC system.

2.2 Tandem Mass Spectrometry (MS/MS)

1. Plant tissue.
2. Methanol (MeOH).
3. 1 N Formic acid (HCOOH).
4. 0.35 N Ammonium hydroxide (NH₄OH).
5. 0.35 N Ammonium hydroxide in 60% Methanol.
6. 0.1% (v/v) Acetic acid.
7. 0.05% (v/v) Formic acid (for mass spectrometry) in Milli-Q H₂O.
8. Extraction buffer.
9. Extraction buffer + deuterated standards.

2.2.1 Equipment

1. Pipet.
2. 96-well Bio-Tubes (1.1 ml tubes) with Caps (8 plug caps), Sealing Mats, and Cap Mats.
3. 96-well Deep Well Plates and 96-well PCR plates.
4. Sealing film.
5. Chroma bond Multi 96 vacuum manifold.
6. Evaporator system (Glas-Col).
7. Tissue homogenizer.
8. Eppendorf Centrifuge 5804 R equipped with a Swing-bucket rotor A-2-DWP (Eppendorf).
9. Ultrasonic bath.
10. Mass spectrometer equipped with a Turbo spray ion source.

3 Methods

3.1 HPLC

Quantification of

Cytokinin (See Note 1)

3.1.1 Plant Extraction and Purification [9]

1. Grind the plant material in liquid nitrogen; extract the samples with 80% methanol, for 12 h at 4 °C in dark.
2. Filter the homogenate and methanol fractions of the extracts passing it through a C18 Sep-Pak cartridge.
3. Evaporate the eluates in vacuum at 40 °C to leave an aqueous phase.
4. Dilute with 9 volumes of 40 mM Ammonium Acetate (pH 6.5), 4 ml of Diethylaminoethyl (DEAE) cellulose equilibrated and centrifuge for 10 min.
5. Now, to remove the DEAE cellulose, pass the supernatant through a C18 Sep-Pak cartridge. Wash the cartridge with 0.1 M Acetic Acid eluted with 6 ml 25% methanol in 0.1 M Acetic Acid (v/v); again, eluted with 10 ml 60% methanol.
6. Let it concentrate to 0.5 ml. Cytokinins can be separated and quantified from this concentrate.

3.1.2 Separation and Quantification of Cytokinins

Perform analysis using HPLC system's isocratic elution program of 30% or 40% methanol for Z/ZR, DHZ/DHZR, and iPA/iP, respectively.

3.2 Tandem Mass Spectrometry (MS/MS) Quantification of Cytokinin (See Note 2)

3.2.1 Preparation of Plant Material [10]

1. Collect the plant tissue and immediately freeze it (−80 °C) in liquid nitrogen.
2. Homogenize the plant tissue under liquid nitrogen.
3. Aliquot 100 mg plant tissue per sample in liquid nitrogen precooled 96-well bio-tubes (with two steel balls per tube, closed with caps). Make small holes in the caps to prevent tubes from exploding.
4. Keep at least for 30 min at −20 °C (for longer storage at −80 °C) before starting with the extraction to evaporate liquefied gases from the tubes.

3.2.2 Plant Extraction and Quantification (See Note 3)

1. Add 800 µl precooled (−20 °C) extraction buffer + deuterated standards to each tube and cover with a precooled sealing mat.
2. Shake in tissue homogenizer for 1 min and incubate over night at −20 °C.
3. After the incubation, shake it in the tissue homogenizer for 1 min and centrifuge (20 min at 4 °C).
4. Collect the 600 µl supernatant in 96-well bio-tubes and keep it at −20 °C.
5. Add 600 µl extraction buffer to the pellet and again shake it in the tissue homogenizer for 1 min.

6. Incubate for 30 min at -20°C , then centrifuge (20 min, at 4°C).
7. Collect 600 μl supernatant and combine it with the supernatant from the **step 1**.
8. Cover the samples with a sealing mat and centrifuge supernatants (20 min, at 4°C) to continue with the sample purification. (In-between the steps, keep the samples on ice)
9. Using HR-X column with 0.6 ml MeOH, discard flow-through, the vacuum manifold to suck the samples and buffers through the column. Same accounts for the following steps.
 - (a) Condition the column with 0.6 ml extraction buffer.
 - (b) Discard flow-through and load the samples to the column (collect flow-through) and subsequent wash with 0.2 ml extraction buffer (collect flow-through).
 - (c) Collection of both flow-through can be done in a Nunc 96-well deep well plate.
10. Evaporate the samples at 45°C under a constant nitrogen stream to remove MeOH from the samples, utilizing the evaporator system. Add 850 μl of 1 N Formic acid (HCOOH) per sample, cover the plate with a sealing mat and shake in the tissue homogenizer for 30s. Centrifuge (20 min, at 4°C).
11. Condition the HR-XC column with 0.6 ml MeOH. Discard flow-through.
12. Condition the column with 0.6 ml 1 M HCOOH . Discard flow-through.
13. Load the samples to the column. Discard flow-through.
14. Wash column with 1 ml of 1 M HCOOH . Discard flow-through.
15. Wash column with 1 ml MeOH. Discard flow-through.
16. Wash column with 1 ml of 0.35 N NH_4OH . Discard flow-through.
17. Elute cytokinin -nucleobases, -ribosides, and -glucosides from the column with 0.35 N NH_4OH in 60% MeOH.
18. Collect flow-through in 96-well bio-tubes.
19. Completely evaporate the samples at 45°C under a constant nitrogen stream using the evaporator system.
20. Reconstitute samples in 50 μl 0.1% (v/v) acetic acid.
21. Cover the plate with a sealing mat and shake in the tissue homogenizer for 60s.
22. Sonicate the samples for 10 min in an ultrasonic bath. Centrifuge (20 min, at 4°C).

23. Fill samples in 96-well PCR plates and cover with the sealing film. Centrifuge (20 min, 4 °C).
24. Proceed to ultra-performance liquid chromatography coupled tandem mass spectrometry analysis.

4 Notes

1. The flow rate will be around 1.2 ml min^{-1} at temperature 30 °C. Peaks may be identified based on their retention times when compared with standards.
2. In extraction method for Tandem Mass Spectrometry (MS/MS), allow MeOH to evaporate until approximately 350 μl of liquid is left, that can be tested with a pipet.
3. Remove the bottom plate of the 96-well rack and place the whole rack in the ultrasonic bath. Do not use 96-well racks without bottom plate for shaking and centrifugation.

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Chapter 35

Ethylene

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Abstract

Ethylene is a plant hormone that is gaseous in nature and popular for its involvement in fruit ripening as well as plant growth and development are influenced by it. Research innovation in this field has made information available to understand its biosynthesis, action of perception, and mechanism. Methods for estimation of ethylene are very well studied for plants in field, greenhouses, laboratory, and storage houses. There are various methods used for ethylene monitoring, detection, and quantification. Each of them has its own merits and demerits; the choice of suitable methods should be driven by the consideration, requirements, and specific application. Gas chromatography is the most widely used technique which is discussed in detail in this chapter.

Key words Ethylene, Plant hormone, Gas chromatography

1 Introduction

A biological molecule with the chemical composition of C_2H_4 , a simple gaseous hydrocarbon is ethylene. It is of utmost importance and popular for its association with fruit ripening but in addition to that it has a profound effect on plant growth and development throughout the life of a plant [1]. It regulates leaf and petal abscission, stress responses, pathogen responses, seed germination, seedling growth, and organ senescence. The regulation of these functionalities is either synergistically or antagonistically with other phytohormones [2, 3]. Gaseous ethylene is released from intracellular space into the environment when the concentration of ethylene is in equilibrium with its dissolved amount in the cytoplasm. L-methionine originated 1-aminocyclopropane-1-carboxylic acid (ACC) is a precursor of ethylene [4]. The change in the production of ethylene in green plants is diurnal and influenced by the temperature [5], nutrient availability [6], light intensity [7], and pathogen attack [8]. It also varies according to plant species, organ type, and plant developmental stage. A closed sampling vessel is necessary to accumulate ethylene. For estimation of ethylene, at

first, Neljubov et al. [9] and Crocker et al. [10] used bioassay protocol and later on replaced by instrumental methods and now direct sampling from cultivation environment [11]. Gas chromatography (GC), electrochemical, and optical sensing are the methods popular these days for the detection. From the perspective of agriculture and industry, optimization of fruit freshness has encouraged the development of sophisticated sensors conjugated with instruments to monitor the concentration of ethylene. The popularity of gas chromatography is due to its sensitivity and user-friendliness.

2 Materials

2.1 Estimation of Ethylene by Gas Chromatography

1. Plant material.
2. Volume container with trapped ethylene in headspace.
3. Ethylene standard solution (10 $\mu\text{L/L}$).
4. Empty headspace vials.

3 Methods

3.1 Estimation of Ethylene by Gas Chromatography (see Note 1)

1. Flow the gas into GC instrument.
2. Turn on the GC instrument and software program.
3. Prepare serial dilution of ethylene standards. It is recommended to prepare more than five standards in serial dilution. Inject each of dilution in blank headspace sealed vials and allow to saturate the headspace with ethylene gas for a while.
4. Place each vial one by one to the instrument and run GC.
5. Open and monitor the real time running the screen of instrument to identify single peak for each standard. Note the retention time of these standards.
6. Similarly, run the samples as per collection.
7. Determine the concentration of ethylene by comparing it with standard curve prepared from the ethylene standards.
8. Divide the concentration by weight of sample per time of incubation to get the unit of the ethylene concentration, i.e., $\mu\text{L/L}$ per gm/day.

4 Notes

1. It is considered that the user has collected the ethylene gas in appropriately sized container from sample plant part by

incubating the sample in closed container for a defined period of time.

2. Please give attention to the sampling procedure and subsequently injection of the sample into the GC column.
3. Choice of carrier gas is depending on user choice and its availability.
4. Use of conventional injection base or concentric rotary valves is of user choice.
5. It is advised to refer various other methods which are not generalized and used for distinct research objective and modes of ethylene detection are listed in Cristescu et al. [12] article.

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Chapter 36

Gibberellin

Dhruti Amin, Sanket Ray, and Abhishek Sharma

Abstract

The phytohormone, Gibberellin (GA) has a regulatory role in seed germination, stem elongation, leaf expansion, pollen maturation, development of flowers, fruits and seeds. Crop breeding programs have increased crop productivity by introducing GA synthesis or response genes to produce high-yielding crops. Plant to plant, species to species, either plant or microbes types, and concentration of GAs vary. Researchers can estimate and identify the type of GAs by spectrophotometric and chromatographic methods.

Key words Phytohormone, Gibberellic acid, Spectrophotometry, Thin layer chromatography

1 Introduction

The plant growth regulator gibberellin A was first isolated from *Gibberella fujikuroi* pathogenic fungus, which causes foolish seedling disease in rice, showing excessive stem symptoms elongation and falling over quickly. Since now, at least 136 fully characterized GAs, named from GA₁ to GA₁₃₆, have been identified from various plants, fungi, and bacteria [1–3]. Many GAs were reported in plants, but few GAs, i.e., GA₁, GA₃, GA₄, and GA₇, act as regulators for plant growth and development [4]. The role of gibberellins is to regulate seed germination, dormancy, stem elongation, flowering, flower development, pollen maturation, and senescence. Looking into the application and function of GAs, the essential requirement is to know the concentration and type of GAs with the agricultural application. The extraction procedure of endogenous and exogenous gibberellin from plants and microbial culture is different [5]. But the quantification procedure is the same for GAs extract irrespective of source.

2 Materials

2.1 Spectrophotometric Method [5, 6]

1. Gibberellin extract.
2. Standard GA solution: 5–50 µg/ml.
3. Zinc acetate solution: Dissolve 21.9 g of zinc acetate in 80 ml distilled water along with 1 ml of glacial acetic acid and make up the volume to 100 ml with distilled water.
4. Potassium ferrocyanide solution (Preparation: Dissolve 10.6 g of potassium ferrocyanide in 100 ml of water).
5. 5% HCl.
6. 30% HCl.
7. Spectrophotometer.

2.2 Colorimetric Method [5, 7]

1. Gibberellin extract.
2. Standard GA solution: 5–50 µg/ml.
3. Phosphomolybdic acid reagent (Preparation: 35 g of 85% molybdic acid and 5 g of sodium tungstate in 200 ml of 10% sodium hydroxide solution and 200 ml of distilled water. Boil the mixture for 40 min (for removal of ammonia present in molybdic acid). Cool the mixture at room temperature and add 350 ml of distilled water followed by 125 ml of concentrated 85% phosphoric acid to the mixture. Final makes up the volume to 500 ml with distilled water).

2.3 Thin Layer Chromatography [5, 8]

1. Gibberellin extract.
2. Benzene.
3. *N*-butanol.
4. Acetic acid.
5. Isopropanol.
6. Water.
7. Ethanol.
8. Concentrated Sulfuric acid.
9. TLC plate.
10. Capillary for sample loading.
11. Hot plate or hot air oven.

3 Methods

3.1 Spectrophotometric method

1. Pipette 15 ml of the test extract and 2 ml of zinc acetate to the test tube, keep the mixture for 2 min at room temperature.
2. Add 2 ml of potassium ferrocyanide.

3. Centrifuge at low speed for 15 min.
4. Take 5 ml of the supernatant, add 5 ml of 30% HCl to it.
5. Incubate the mixture at 20 °C for 75 min.
6. Take 5% HCl as blank.
7. Measure the absorbance of the blank and test samples at 254 nm in a spectrophotometer.
8. Calculate the amount of GA present in the extract from a standard curve prepared with known GA₃.

3.2 Colorimetric Method

1. Take 1 ml of extract and 15 ml of phosphomolybdic acid reagent into 25 ml volumetric flasks.
2. Mix the content thoroughly and place the flask in a boiling water bath for 1 h.
3. After incubation, remove and place the flask immediately in an ice water bath.
4. Make up the volume to 25 ml with distilled water.
5. Measure the absorbance at 780 nm in a colorimeter using distilled water blank.
6. Maintain reagent blank and subtract the absorbance of the reagent blank from the values obtained with samples.

3.3 Thin Layer Chromatography

1. Load the test sample and standard GA₃ with the help of a capillary on the TLC plate.
2. Develop the chromatogram in a benzene-*n*-butanol-acetic acid (30:15:5, v/v) or di-isopropyl ether-acetic acid (95:5, v/v) or isopropanol-water (4:1, v/v).
3. Spray with either ethanol-conc. H₂SO₄ (95:5, v/v) or Water-conc. H₂SO₄ (30:70, v/v).
4. Heat the chromatograms at 120 °C for 10 min in a hot air oven or on the hot plate.
5. Remove the plate, cool and examine under UV light for fluorescent spots (*see* **Notes 1** and **2**).

4 Notes

1. Gibberellins such as A₁, A₃, A₅, A₆, and A₈ will appear as blue fluorescent spot on TLC plate after ethanol-conc. H₂SO₄ spray under UV light due to the presence of 7 hydroxyl group [5].
2. Gibberellins such as A₁, A₃, A₆, and A₈ will appear as blue-green fluorescent spot while A₂, A₄, and A₉ appear as blue-purple spots; A₅ and A₇ as light yellow and brown fluorescent spot respectively on TLC plate after water-conc. H₂SO₄ spray under UV light [5].

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Chapter 37

Brassinosteroids

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Abstract

Plant uses extrinsic factors like air, water, light, and nutrition for the regulation of growth and development. Likewise, it also uses intrinsic factors which are phytohormones, active molecules produced by plant cells vital for embryogenesis, abiotic stress, biotic stress, and reproductive development. From classified hormones, the brassinosteroids (BRs) are one of the novel plant hormone groups of isoprenoid origin. They are regarded as hormones with pleiotropic effects because they exhibit remarkable growth supporting property. Apart from its normal functioning of cell growth, cell elongation, photomorphogenesis, and seed germination, it also helps plants to mitigate the environmental biotic and abiotic stress by acting as stress-reliever.

Key words Phytohormones, Brassinosteroids, Chromatography

1 Introduction

Phytohormones are the small molecules released by the plant cells in very low concentrations (ng-μg/kg FW). Each plant cell is capable of producing plant hormones, unlike the animal cells where the production of the hormone is limited to certain glands. Plant with the help of hormones controls various aspects like growth, development, reproduction, disease resistance, biotic stress, and abiotic stress. The hormones act on gene expression and transcription and ultimately influence cell growth, development, and differentiation. Within plants, the produced hormone molecules transport through cytoplasmic streaming, phloem, and xylem. According to chemical structure and their physiological effect, the plant hormones are classified into abscisic acid, auxins, cytokinins, ethylene, gibberellins, brassinosteroids (BRs), jasmonates, salicylic acid, and strigolactones.

Polyhydroxysteroids are the only steroid-based hormones that are classified as BRs in plants and are essential for both growth and development [1]. In the year 1979, brassinolide was identified as BR for the first time and was isolated from extracts of *Brassica napus* pollen [2] and later on from bean, corn, sunflower,

buckwheat, and orange pollens [3, 4]. It controls gravitropism, xylem differentiation, resistance to stress, cell elongation, and division. Additionally, they also inhibit leaf abscission and root growth. They increase DNA and RNA polymerase activity, induce cell elongation and cell division, kindle ethylene production, network synergistically with auxins and elevate tolerance to abiotic stress [5–7]. BRs can help plants to tolerate abiotic stress like cold, heat, salinity, and drought. It is observed that such a type of tolerance of BRs is associated with the expression of RD29A and ERD10 genes [8]. Structurally, BRs are derived from the carbon skeleton of 5- α -cholestane and substitution of A-ring, B-ring, and C-17 side chains create structural variants [9, 10].

Bioassays and gas chromatography-mass spectrometry is the most widely used technique among different techniques developed for the estimation of BRs in plant tissues. The analytical method used for the separation and analysis of BRs is high-performance liquid chromatography (HPLC) [4]. As BRs have no chromophore for detection, with the help of pre-labeling reagents they are often derivatized to make them responsive to detection. Several reagents like 9-phenanthreneboronic acid, ferroceneboronic acid [11], boronic acid [12], phenylboronic acid [13], and 1-cyanoisoin-dole-2-m-phenylboronic acid are used for derivatization of BRs. These derivatized BRs are well separated on HPLC with octadecyl silica gel column using a mobile phase of acetonitrile-water mixtures. Gamoh et al. [14] also analyzed derivatized BRs using the atmospheric pressure chemical ionization (APCI) based method of liquid chromatography/mass spectroscopy (LC-MS) with reversed-phase HPLC while Svatos et al. [15] used liquid chromatography-electrospray ionization-mass spectroscopy (LC-ESI-MS). The bioassay-based protocol used ELISA and antibodies developed against BRs. There are reports on the development of monoclonal antibodies against BRs [16]. Owing to their significance and important role in plants, BRs are now much of interest in agriculture.

2 Materials

2.1 Analysis of BRs Using Thin Layer Chromatography

1. BRs standard: 24-Epibrassinolide.
2. Extracted BRs samples from plant.
3. Silica gel 60 F-254 coated TLC plates.
4. Chloroform.
5. Methanol.

6. UV light 254 nm.
7. Liebermann–Burchard reagent.

**2.2 Analysis of BRs
Using High-
Performance Liquid
Chromatography**

1. *m*-Dansylaminophenylboronic acid (DABA).
2. Acetonitrile.
3. Pyridine.
4. HPLC with a fluorometric detector.
5. Reverse phase column with octadecyl group as stationary phase; diameter: 6.0 mm i.d.×15 cm.
6. Mobile phase: acetonitrile: water (80:20).

3 Methods

**3.1 Analysis of BRs
Using Thin Layer
Chromatography**

Preliminary and qualitative analysis of BRs.

1. Prepare mobile phase by mixing chloroform and methanol in 88:12 combination.
2. Procure readymade Liebermann–Burchard reagent or prepare manually by mixing ice cold 220 ml of acetic anhydride and 200 ml glacial acetic acid followed by addition of 30 ml ice cold sulfuric acid to this mixture. Reagent is stable for 6 months if stored at 4 °C. If stored at room temperature, the darkening will show the deterioration of reagent [17].
3. Prepare appropriate dilution of 24-Epibrassinolide in ng/ml or pg/ml (*see Note 1*).
4. It is advised to pre-run the TLC plates with methanol and heat dry for better performance.
5. Spot the standard and sample on TLC plate and run the plate with prepared mobile phase of chloroform: methanol (88:12).
6. Let the plate air dry and spray the Liebermann–Burchard reagent on the plate to detect the samples.
7. Calculate the *R_f* values and compare the *R_f* values of standard and samples.

**3.2 Analysis of BRs
Using High-
Performance Liquid
Chromatography**

1. Prepare DABA solution by dissolving 10 mg DABA in 1 ml of 0.1% (v/v) pyridine in acetonitrile.
2. For derivatization of extracted BRs, mix 100 µl of DABA solution to BRs sample extract (1:1) and heat at 70 °C for 10 min. Cool the reaction and use it for HPLC analysis.
3. Inject the derivatized sample in reverse phase column and run with mobile phase acetonitrile: water (80:20) with the flow rate of 1.0 ml/min.

4. Verify the dansyl fluorophore in the derivatized product with fluorometric spectral data at a scan rate of 60 nm/min in aqueous acetonitrile.
5. Determine the maximum excitation and emission spectra and quantify the concentration of BRs in samples.

4 Notes

1. In thin layer chromatography, methanolic extract from plant can also be applied as sample to detect the presence of BRs.
2. In thin layer chromatography, water can be used in the mobile phase to achieve better separation.
3. Other derivatization reagent can be used for detection of BRs as reported in literature [18].
4. No commercial antibody is available for plant tissue to date so method for estimation of BRs using immunoglobulin is advisable.

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Strigolactones: Extraction and Characterization

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Abstract

Strigolactones (SLs) have recently discovered an important group of plant hormones. Strigolactones are biosynthesized in roots followed by transportation to shoot or release into the rhizosphere. They are involved in root development, plant-microbes interaction in rhizosphere by promoting growth of arbuscular mycorrhiza fungi, also providing mineral nutrients, nitrogen fixation and nodulations in legumes. Analysis of strigolactones is challenging because of its low amount (15–30 pg/plant/day) and interference created by primary metabolites, co-presence of structural analogs and highly complex plant matrix. The current analytical procedures adopted for strigolactones isolation, purification, and characterization are non-specific, time consuming with absolute requirement of high sample amount. Development of simple, rapid, reliable, and accurate protocol for strigolactones analysis will be very useful to initiate further research on different aspects of this hormone. The source for strigolactones isolation (root exudates and root tissue) are important factors for further selection of analytical methods. Solvent extraction, solid phase extraction, high performance liquid chromatography (HPLC), gas chromatography (GC), mass spectrometer (MS) are some of the important methods associated with strigolactones analysis. Some new tools such as HR-DART-MS (direct analysis in real time) and DESI-MS (desorption electrospray ionization) reported to reduce sample size significantly and yield results directly using crude extract.

Key words Plant hormones, Plant exudates, Rhizosphere signaling, HPLC, LC–MS

1 Introduction

Research related to identification of plant secondary metabolites and small molecules such as hormones are important for deciphering impact on plant development and association with other biotic components. Strigolactones (SLs) is nowadays a very favorite topic in plant hormone research [1–3]. Strigolactones (SLs) is a group of plant hormones widely distributed in the plant kingdom. Diverse mono, dicotyledonous plants have been reported to produce SLs [3]. The first SLs was isolated from cotton and named as strigol after the name of parasite (striga) present on cotton plant [4]. The structure of SLs consists of tricyclic lactone and methylated

butenolide; different modification in tricyclic lactone such as hydroxylation and acetylation affected biological activity of SLs [5].

More than twenty natural SLs have been isolated from different plants [6]. Orobanchol, solanacol, strigol, heliolactone, avenaol, zealactone, and zeapyranolactone are some of the SLs. Synthesis of SLs can be constitutive and inducible [2]. They are best known for interactions with parasitic plants (Striga, Orobanche, Phelipanche) responsible for decreasing the yield of agriculture crops [3, 7]. The seeds of these parasitic plants germinate only when they receive chemical signals in the form of SLs produced and released from the roots of host and nonhost plants [8]. Because of this reason, SLs are also termed as “germination stimulants.”

As plant hormones, SLs regulate developmental processes including the induction of secondary growth, acceleration of leaf senescence, stimulation of internode growth, and root elongation. SLs also inhibit axillary bud outgrowth and the formation of adventitious and lateral roots [9]. Arbuscular mycorrhizal fungi have obligatory mutualistic interactions with many plants. Presence of SLs such as sorgolactones and strigol helps in increasing hyphal branching [10].

SLs can be obtained from root tissue and root exudates. In comparison with a significant amount of other plant hormones, very low numbers of SLs are found in root exudates. In the initial stages, several liters of root exudates were used for SLs isolation by charcoal and polystyrene-divinylbenzene based resins followed by solvent elution [11]. Presence of low phosphorus (P) in soil reported to intensely increase the biosynthesis of SLs, making its detection easy [11]. SLs may be deactivated during the purification process because of their sensitivity to hydrolysis in an aqueous medium [3, 12].

Conventional HPLC systems are largely replaced by UHPLC (ultrahigh performance liquid chromatography), which offers short analysis times and greater resolution. The most routinely used technique for final analysis of SLs is liquid chromatography-mass spectrometry, which combines a high-resolution chromatographic system with sensitive and acceptably selective mass spectrometric detection [12].

2 Materials

Plant materials—*Glycine max*, *Phaseolus vulgaris*, *Vicia faba*, *Arachis hypogaea*, *Astragalus sinicus*, *Medicago sativa*, *Pisum sativum*, *Trifolium incarnatum*, *Vigna angularis*, *Oryza sativa*, *Solanum lycopersicum*, *Orobanche minor* seeds, and others.

2.1 Extraction of Root Exudates

1. Selected plant root exudate solution.
2. Activated charcoal, MgSO_4 , K_2HPO_4 .
3. Acetone, ethyl acetate.

2.2 Extraction of Root Tissue

1. Selected plant root.
2. Ethyl acetate.
3. Liquid nitrogen.
4. Ultrasonic bath.
5. Centrifuge.
6. Rotavapor.
7. Mortar and pestle.

2.3 Germination Assay for Qualitative Presence of SLs

1. Root exudate or root tissue extract.
2. Methanol.
3. Petri dishes.
4. Filter paper.
5. Standard solution of synthetic SLs.

2.4 HPLC Method for SLs Estimation

1. Root tissue and root exudate extract.
2. ODS (C_{18}) column.
3. Methanol.
4. SLs standard—Strigol, (+)-orobanchol, GR24.

2.5 UHPLC-MS/MS Method for SLs Characterization

1. Solvents—Deionized water, methanol, formic acid.
2. SLs standard—Strigol, (+)-orobanchol, GR24.
3. Ultrasonic bath, rotavapor.

3 Methods**3.1 Extraction of Root Exudates [13]**

1. Grow the plants according to Yoneyama et al. [13] in absence of phosphorous.
2. Take sufficient quantity (around 1 l) of root exudate from about 20 to 100 plants.
3. Adsorb root exudates on charcoal and elute it with acetone.
4. Evaporate acetone under vacuum and dissolved in 50 ml water.
5. Extract three times with 50 ml ethyl acetate and washed with 0.2 M K_2HPO_4 (pH—8.3), dried over anhydrous MgSO_4 and concentrated under vacuum.
6. Store the extract in sealed glass vials at 4 °C until use.

3.2 Extraction of Root Tissue [1]

1. Grow the plant in absence of phosphorous.
2. Take about 100–1000 mg of roots and ground in mortar using liquid nitrogen.
3. Extract with 10 ml ethyl acetate in ultrasonic bath for 10 min.
4. Centrifuge for 10 min at 5000 rpm ($1674 \times g$).
5. Transfer organic phase to glass vials.
6. Repeat the procedure thrice and concentrate in rotavapor, store at -80°C .

3.3 Germination Assay for Qualitative Presence of SLs [14]

1. Take aliquot of root exudate or root tissue extract in 60% methanol.
2. Prepare separate standard solution of synthetic SLs (orobanchol, strigol, and 5-deoxystrigol).
3. Dispense test and standard to 5 cm petri dish lined with filter paper.
4. Put the Orobanche minor seeds (washed with milli-Q water) on filter paper.
5. The Petri dishes are sealed, enclosed in polyethylene bags, and placed in the dark at 23°C for 5 days.
6. Seeds are considered germinated when the radicle protruded through the seed coat.
7. Seed germination indicates presence of SLs in extract.

3.4 HPLC Method for SLs Estimation [8]

1. HPLC parameters: Mobile phase—60% methanol in water for 30 min and 100% methanol after 30 min, ODS (C_{18}) column, Flow rate—0.2 ml/min, column temperature— 40°C , Injection volume—10 μl . Decide run time based on peak elution profile.
2. Dissolve the crude extract with 60% methanol (to get final concentration of 10 $\mu\text{g}/\text{l}$).
3. Estimate the SLs in the root exudates by comparing retention times of germination stimulants with those of synthetic (or natural) SLs.
4. Wash the column for 20 min with 100% methanol after analysis.

3.5 UHPLC-MS/MS Method for SLs Characterization [1, 3]

1. UHPLC parameters: Injection volume—5 μl , C_{18} (100 mm $\times$ 2.1 mm, 1.7 μm particle size), Flow rate—0.3 ml/min., column temperature— 40°C , mobile phase—solvent A (water, 0.1% formic acid) and solvent B (MeOH, 0.1% formic acid).
2. Optimized linear gradient program: 0–0.5 min, 50% B; 0.5–5 min, to 100% B; 5–7 min, 100% B; 7–7.5 min, to 50% B; 7.5–10.5 min, 50% B.

3. Autosampler temperature 5 °C.
4. Use triple quadrupole mass spectrometer with an electrospray ionization (ESI) source in positive mode.
5. MS parameter: spray voltage +4500 V, cone temperature 300 °C, cone gas flow 15 psi, heated probe temperature 400 °C, heated probe gas flow 15 psi, nebulizer gas flow 55 psi, and collision pressure 2.0 mTorr.

4 Notes

1. It is useful to pre-filter the extract sample through guard column to decrease the interference created by sample matrix.
2. Use freshly prepared test and standard solutions.
3. Mobile phase can be used for 1 week but do not use if the visible precipitate/turbidity observed.
4. Root exudate can be obtained from hydroponically grown plant.
5. Interpret the MS data using software and from previously published literature.

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Estimation of Jasmonic Acid Using Non-pathogenic Microbes *Jasmonic Acid*

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Abstract

Jasmonic acid (JA) is a well-known lipid-derived signaling molecule that is produced by certain bacteria, fungi, and plants. It plays an important role in the induction of Induced Systemic Resistance (ISR) in plants. The biosynthesis and function of JA from plants has been well documented, but the information on this molecule is still scarce for microorganisms. This chapter summarizes the knowledge on the occurrence of jasmonates from plants as well as microbial culture media and the culture conditions for optimal JA production as an alternative source for the production of these valuable metabolites.

Key words Jasmonic acid, ISR, *Lasiodiplodia*, Soybean, HPTLC, GC

1 Introduction

Jasmonic acid is an important signaling molecule produced by plants as well as many microorganisms. It is biosynthesized from **linolenic acid**, which is oxygenated by **Lipoxygenase** (13-LOX), forming a peroxide. This peroxide then cyclizes in the presence of allene oxide synthase to form an allene oxide. This allene oxide is acted upon by the enzyme allene oxide cyclase which forms 12-oxo phytodienoic acid. This compound further undergoes a series of β -oxidations to 7-iso-jasmonic acid. In the absence of enzymes, this iso-jasmonic acid isomerizes to jasmonic acid [1].

Induced systemic resistance (ISR): The plant root colonizing microorganisms such as plant growth promoting fungi (PGPF) or plant growth promoting rhizobacteria (PGPR) help in suppressing plant disease incidence as they trigger defense mechanisms in plant [2, 3]. The induction of ISR by PGPR or PGPF differ from Systemic Acquired Resistance (SAR) for their signal transduction pathways [4, 5]. The signalling molecules, Jasmonic acid (JA) and Ethylene (ET) help in induction of ISR and for accumulation of defense related enzymes, while SAR helps in PR protein expression

in response to induction by the signalling molecule, Salicylic acid [6].

Plant Growth Promoting Rhizobacteria (PGPR): Root colonizing non-pathogenic bacteria are generally called as plant growth promoting rhizobacteria (PGPR). They are of two types: extracellular plant growth promoting rhizobacteria (ePGPR) and intracellular plant growth promoting rhizobacteria (iPGPR) [7]. The PGPRs exist in the rhizosphere, on the rhizoplane and in the spaces between the cells of the root cortex, while iPGPR reside within a specialized nodular structure in plants. Some commonly reported PGPR genera which show plant growth promoting activities are *Pseudomonas*, *Azospirillum*, *Azotobacter*, *Bacillus*, *Burkholderia*, *Enterobacter*, *Rhizobium*, *Erwinia*, *Mycobacterium*, *Mesorhizobium*, *Flavobacterium*, *Pseudomonas fluorescens*, and *Pseudomonas syringae* [8–13].

Plant growth promoting fungi: The non-pathogenic fungi which colonize the plant roots are known as plant growth promoting fungi (PGPF). They have been found to play an important role in triggering induced systemic resistance in cucumber and in the effective control of soil-borne diseases like damping-off caused by *Fusarium*, *Rhizoctonia*, and *Sclerotium* and take-all caused by *Gaeumannomyces graminis* of a number of crop plants [14–16].

2 Materials

2.1 Production and Extraction of Jasmonic Acid from Bacterial Strain

1. *E. coli* RC 424.
2. Luria broth.
3. YEM.
4. HCl.
5. Chloroform.
6. Ethyl acetate.
7. Methanol.
8. Acetic acid.
9. Jasmonic acid standard.
10. *N,N*-diisopropylethylamine.
11. Pentafluorobenzyl bromide.
12. *n*-hexane.
13. Diethyl ether.
14. Acetonitrile.

2.2 Production and Extraction of Jasmonic Acid from Fungal Strain

1. *Lasiodiplodia theobromae* strain.
2. Potato dextrose agar.
3. Basal salt broth.
4. 6 N HCl.
5. Ethyl acetate.
6. Isopropanol.
7. Ammonia.
8. Jasmonic acid standard.
9. Methanol.
10. Formic acid.
11. Milli Q water.
12. TLC plates.
13. Mortar and pestle.
14. Erlenmeyer flask.
15. Petri plates.

2.3 Production and Extraction of Jasmonic Acid from Glycine Max

1. Acetone.
2. 100 mM potassium phosphate.
3. 6 M HCl.
4. DEAE cellulose.
5. Chloroform.
6. Hexane.
7. Ethyl acetate.
8. Acetic acid.
9. Diazomethane.
10. Dichloromethane.

2.4 Determination of Jasmonic Acid and Methyl Jasmonate from *Ulmus pumila* Leaves by GC-MS

1. Jasmonic acid.
2. Methyl jasmonate.
3. Leaves of *U. pumila* at different infection stages including galls formation stage and dehiscence.
4. Liquid nitrogen.
5. Methanol.
6. Dihydro jasmonate (DHJA).
7. Ethyl acetate.

3 Method

3.1 *Production and Extraction of Jasmonic Acid from Bacterial Strain [17, 18]*

Extraction of jasmonic acid from bacterial strains and analysis using HPLC

1. Acquire appropriate bacterial strain (e.g., *E. Coli* RC 424 Strain) from microbial type culture collection centers.
2. Grow bacterial culture strain for 72 h in 25 ml of Luria broth media at stationary phase or YEM at 36 ± 1 °C.
3. The control media should contain 10^{10} cfu/ml cell density while 10^8 cfu/ml in media with 13.7% polyethylene glycol.
4. Take bacteria grown in media and centrifuge the bacterial cultures at 8000 rpm, 4 °C for 20 min.
5. Collect the supernatant and use HCl solution to acidify the sample supernatant, i.e., pH 2.5.
6. Partition the sample either by using 30 ml of chloroform or 25 ml ethyl acetate.
7. Collect the partitioned sample and evaporate to dryness.
8. Re-dissolve the sample extract residue in 10 ml of solvent.
9. Purify the extract on DEAE-Sephadex A 25. Wash the columns with 3 ml methanol followed by 3 ml 0.1 M acetic acid in methanol.
10. Collect the eluate with 3 ml 1 M acetic acid in methanol and 3 ml 1.5 M acetic acid in methanol.
11. Evaporate the eluate and purify on preparative high-performance liquid chromatography (HPLC) system.
12. Elution rate is fixed to 2 ml/min with a gradient of 10–73% (v/v) methanol in 1% (v/v) acetic acid for 30 min, until 73% (v/v) methanol concentration is reached.
13. Collect 2 ml of sample fraction corresponding to the retention time of jasmonic acid standard.

Estimation of jasmonic acid using gas chromatography

1. For gas chromatography, dissolve the samples in 200 μ l CHCl_3 /*N,N*-diisopropylethylamine (1:1) and derivatize it with 10 μ l pentafluorobenzyl bromide overnight at 20 °C.
2. Take a derivatized sample and dissolve it in 7 ml *n*-hexane and pass through a Chroma bond SiOH column.
3. Elute pentafluorobenzyl esters with 7 ml *n*-hexane /diethyl ether (2:1).
4. Collect the eluent and evaporate it and re-dissolve it in 100 μ l acetonitrile (MeCN).
5. Carry out the analysis using gas chromatography-mass spectrometry (GC-MS).

3.2 Production and Extraction of Jasmonic Acid from Fungal Isolate (See Notes 1–9)

1. *Lasiodiplodia theobromae* (*L. Theobromae*) (MTCC 3068) is procured from microbial type culture collection centers.
2. Grow *L. Theobromae* on potato dextrose agar plate and incubate at 30 °C for 48 h.
3. Excise agar plug and transfer in 250 ml Erlenmeyer flask containing 100 ml of Basal salt broth.
4. Now incubate the flask for 7 days at 27 °C $\pm$ 1 °C in static submerged condition.

A. *Mycelia for morphological identification:*

1. Collect small amounts of fungal mycelia and carry out microscopic observation for morphological identification of given strain.

B. *Crude Mycelia for enzymatic analysis:*

1. The collected mycelium is dried overnight in the oven at a constant temperature of 40 °C.
2. Take 1 g of dried mycelia and homogenize it with 4 ml of 10 mM potassium phosphate buffer (pH 7.4) containing 0.5 mM PMSF in pre-chilled mortar and pestle.
3. Centrifuge at 4 °C at 7000 rpm for 20 min.
4. Collect the supernatant and use this extract for LOX, AOX, and OPR assay.

C. *Extraction of Jasmonic acid* [19]:

1. Take 100 ml of fungal culture filtrate, centrifuge and collect the supernatant and filter using filter membrane.
2. Acidify the filtrate to pH 3 with 6 N HCl.
3. In a separating funnel, add filtrate and ethyl acetate in equal volume, shake well and let it stand for some time. Repeat this method at least three times.
4. Final organic phase is collected from separating funnel and evaporated to dryness under the flow of N₂.
5. Concentrate the collected jasmonic acid in 1 ml ethyl acetate and use it for further HPTLC analysis.

D. Identification and quantification of jasmonic acid:

Estimation of jasmonic acid using HPTLC

1. Take preconditioned TLC plates aluminum backed of 0.25 mm thickness and run with methanol as a mobile phase.
2. Dry the TLC plate in the oven at 120 °C for 20 min.
3. Load the sample (concentrate extracted and re-dissolve in ethyl acetate) using Linomat 5 applicator.

4. Load the sample 10–20 μL and standard of jasmonic acid with different concentrations (30–60 μg).
5. Use isopropanol-ammonia-water [10:1:1(v/w)] solvent system to resolve the bands. Development time is approximately 90 min.
6. Dry the TLC plate in the oven at 50 °C.
7. Detect jasmonic acid sample bands and compare them with standard jasmonic acid.
8. Use a TLC scanner, for quantification of jasmonic acid. Scan the TLC plate at 295 nm.
9. Use software to calculate the exact amount of jasmonic acid present in a fungal sample.

Estimation of jasmonic acid using HPLC technique [20]

1. The ethyl acetate extract is evaporated and re-dissolved in 1.5 mL of mobile phase, filtered (0.45 μm) and analyzed by HPLC–MS/MS.
2. Take 20 μL of samples and standard, filter them through 0.22 μm HPLC filters.
3. Inject the samples/standard in C18 reverse-phase Zorbax SB-C18 HPLC column (3.5 μm , 2.1 $\times$ 150 mm, Agilent Technologies) held at 40 °C.
4. For chromatographic separation, use mobile phase A (consisting of 0.1% formic acid (w/v) in Milli Q water) and mobile phase B (consisting of 0.1% formic acid (w/v) in methanol).
5. Elute at a flow rate of 200 $\mu\text{L}/\text{min}$ during the whole separation.
6. Carry out the analysis on a HPLC/MS system using an electrospray (ESI) interface.
7. Operate the mass spectrometer in the positive mode with a capillary spray voltage of 3500 V, and a scan speed of 22,000 (m/z)/s from 50 to 400 m/z .
8. Analyze the data using appropriate software.

3.3 Extraction and Quantification of Jasmonic Acid from Plants [21]

1. Plant Material: Grow Soybean [*Glycine max* (L.) Merr. cv. Williams] seedlings in darkness at 28 °C and 100% relative humidity. Excise the tissue sections under a green safelight and immediately freeze in liquid N₂ and store at –80 °C until extracted.
2. Homogenize the plant tissue in acetone, filter and add 374 ng of 2-(+)-[13C] JA.
3. After addition of 40–50 ml of distilled water, rotary-evaporate the extracts to the aqueous phase.
4. Bring the samples to 100 mM potassium phosphate (pH 7.5) and lower the pH of the extract to 2.5 with 6 M HCL.

5. Add a suspension of DEAE cellulose [100 mM potassium phosphate (pH 2.5)] and filter the extract. This step permits the removal of the bulk of the chlorophyll in the samples.
6. Partition the samples three times against equal volumes of chloroform.
7. Dry the chloroform phase over anhydrous sodium sulfate and remove by rotary evaporation.
8. Dissolve the crude extract in a small volume of 90% hexane/10% ethyl acetate/1% acetic acid and purify by using a Whatman amino column (4.6×216 mm) with a flow rate of 1 ml/min.
9. Hold the solvent composition at initial conditions (90% hexane/10% ethyl acetate/1% acetic acid) for 5 min followed by a linear gradient to 30% hexane/70% ethyl acetate/1% acetic acid in 10 min. Hold all solvent compositions at these final conditions for 15 min.
10. Collect, dry, and methylate the fractions corresponding to JA (26–28 min) by using ethereal diazomethane.
11. After methylation, purify the samples further by using a Waters silica gel column (3.9×150 mm) with a flow rate of 1.5 ml/min. The initial solvent composition is 100% hexane with a gradient to 90% hexane/10% ethyl acetate in 5 min. Hold the solvent composition at these final conditions for 10 min.
12. Collect and dry the fraction corresponding to JAME (12–13 min). Dissolve the fraction in 20 ml of dichloromethane. Analyze it by GC/MS/selected ion monitoring, at m/z 83, 95, 151, 224, and 225 with a dwell time of 100 msec for each ion.
13. Make corrections for the natural abundance of stable isotopes by using a calibration curve with various amounts of unlabeled and [^{13}C] JA or by using a modification of the isotope dilution equation.
14. Since 7-iso-JA can isomerize to JA during extraction, peak areas corresponding to these two isomers are combined to give a total amount of both isomers. Generally, JA and 7-iso-JA are present at a 9:1 ratio.

3.4 Determination of Jasmonic Acid and Methyl Jasmonate from *Ulmus pumila* Leaves by GC-MS [22] (See Note 10)

Preparation of Standard Solutions:

1. Approximately 10 mg of individual standards, of jasmonic acid, and methyl jasmonate to be dissolved in methanol.
2. Prepare the mix working solutions by diluting the appropriate volume of standard stock solutions with methanol. Store the stock solutions and working solutions in dark at -20°C freezer.

Sample Preparation:

1. Place all the leaves in liquid nitrogen at room temperature.
2. Let the liquid nitrogen evaporate, weigh 1.0 g of samples into a 50 mL into a centrifuge tube, and then add 10 μ L internal standard of dihydro jasmonate (DHJA) and 10 mL of 80% cooled methanol (pH 2.5–3.0).
3. Homogenize the mixture using a blender for 2 min at 6000 rpm and then soak overnight at 4 °C.
4. The next day, add 10 mL ethyl acetate into the tube and vortex for 1 min and then centrifuge for 10 min at 10,000/min at 4 °C.
5. Collect the supernatant and add 0.2 g GCB and 0.6 g PSA, and then vortex for 1 min.

GC-MS Conditions:

1. Inject the extract into an Agilent 6890N-5973i GC/MS equipped with a DB-5MS (UI) chromatographic column (30 m $\times$ 0.25 mm $\times$ 0.25 μ m).
2. Set the injection temperature and injected volume to 280 °C and 2 μ L, respectively.
3. The helium flow rate would be 1.1 mL/min.
4. The temperature program is as follows: the initial column temperature to be set at 70 °C for 4 min, increasing to 300 °C at the speed of 10 °C/min and lasting for 2 min and then increasing to 340 °C at the speed of 5 °C/min and holding until the end of analysis.
5. Perform the quantification in the selected-ion monitoring (SIM) mode after electron ionization (70 eV) with a dwell time set at 0.3 s, and set the source temperature and quadrupole temperature at 230 °C and 150 °C, respectively.
6. Collect the mass data in the range from m/z 40 to m/z 500.

4 Notes

1. Composition of potato dextrose agar g/L: infusion from potatoes, 200; Dextrose, 20; Agar, 15; pH 5.6 ± 0.2 .
2. Composition of Basal salt broth g/l: sucrose, 50; Malt extract, 19.45; NaNO₃, 7.5; KH₂PO₄, 2.0; KCl, 0.6; MgSO₄·7H₂O, 3.5; FeSO₄·7H₂O, 0.6; ZnSO₄·7H₂O, 0.003; MnSO₄·7H₂O, 0.1; CuSO₄·7H₂O, 0.003; Na₂MoO₄·2H₂O, 0.003; yeast extract, 1.0; pH 5.5.
3. Lactophenol cotton blue stain can be used for morphological identification of fungi.
4. To support the synthesis of jasmonic acid, LOX, AOS, and OPR enzymes involved in the jasmonic acid pathway should be analyzed.

5. LOX activity can be measured using UV spectrophotometrically at 234 nm as an appearance of conjugated diene.
6. AOS enzyme activity can be carried out by using HPLC C18 reverse-phase column technique.
7. OPR enzyme assay can be confirmed using HPLC.
8. The crude mycelia extract can be profiled for protein using SDS gel [23].
9. In HPTLC technique, other solvent system like {benzene-ethyl acetate [1:1 (v/v)]; chloroform-ethyl acetate [1:1 (v/v)]; chloroform-methanol [9:1 (v/v)]; and diethyl ether} can be studied for resolving bands of jasmonic acid on TLC plate.
10. In GCB and PSA, after centrifugation at 5000 rpm/min for 5 min, collect and evaporate the supernatants to dryness with a gentle stream of nitrogen and then dissolve in 1 mL ethyl acetate for further analysis.

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Part IV

Screening of Agriculturally Important Enzymes



Chitinase

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Abstract

Chitinases are glycosyl hydrolases with sizes ranging from 20 kDa to about 90 kDa. They are present in a wide range of organisms such as bacteria, fungi, yeasts, plants, actinomycetes, arthropods, and humans. Chitinases can degrade chitin directly to low molecular weight chitooligomers, which serve a broad range of industrial, agricultural, and medical functions such as elicitor action and anti-tumor activity. Chitin, a linear polymer of β -1, 4- *N*-acetylglucosamine (GlcNAC), is the second most abundant biopolymer on the planet. Chitin is found in the outer skeleton of insects, fungi, yeasts, algae, crabs, shrimps, and lobsters, and the internal structures of other invertebrates. Chitinases are a huge and diverse group of enzymes that show differences in their molecular structure, substrate specificity, and catalytic mechanism. It is vital to study the substrate specificity of chitinases as it not only reveals the relationship between the substrate specificity and physiological roles but also allows one to degrade chitin into novel products having industrial applications. Chitin is the second most abundant polysaccharide after cellulose and is mostly found in the cell wall of fungi, yeast, exoskeletons of insects, and crustacean shells. There has been a steady increase in demand for chitin derivatives, obtained by the action of chitinases on chitin polymer for various industrial, clinical, and pharmaceutical purposes. Hence, this chapter focuses on the properties and applications of chitinases.

Key words Chitinases, Hydrolytic enzyme, Chitin, Biocontrol

1 Introduction

Chitinases have been receiving increased attention due to their role in the biocontrol of fungal phytopathogens [1] and harmful insects [2]. Of late, chitinases have also attained a lot of attention as they are thought to play a key role in mosquito control and plant defense systems against chitin-containing pathogens [2]. Chitin and chitinases are used by pathogens (mainly protozoan or metazoan) causing animal and human diseases. Several pathogens contain chitin coats, giving them protection against both external and internal (in a host) environment. Others attack their host using chitinase. To establish a successful infection or transmission from one vertebrate to another, they exploit the chitin-containing structures of the host [3]. Several bacteria can produce chitinases,

including *Streptomyces* [4], *Alteromonas* [5], *Escherichia* [6], *Aeromonas* [7]. Chitinase-producing bacteria have been isolated from soil, shellfish waste, garden and park waste compost, and hot springs [8]. The chitinases of the two different families do not share amino acid sequence similarity and have completely different 3-dimensional (3D) structures and molecular mechanisms. Therefore, they are likely to have evolved from different ancestors.

Designing of chitinases by applying directed laboratory evolution and rational approaches for improved catalytic activity for cost-effective field applications has also been explored. However, there are immense challenges for the production of specific and cost-effective enzymes [9]. In the future, there is a possibility of generating chitinases with novel functions. Chitinases can be exploited for their use as food preservatives, thereby increasing the shelf life of the foods. A vast understanding of the biological roles of different chitinases would help us to develop novel therapeutic approaches for several diseases including asthma, and chronic rhinosinusitis. There is a possibility of using chitinases as antitumor drugs since chitohexaose and chitoheptaose have shown anti-tumor activity. These enzymes can be used for the enhancement of the human immune system [10].

2 Materials

2.1 Isolation and Identification of Chitinase-Producing Bacteria

1. Microorganism: bacterial strain.
2. Nutrient agar (supplemented with sucrose/glucose).
3. Broth medium.
4. Distilled water.
5. Sugar tubes.
6. Pipette.
7. Distilled water.
8. Sugar tubes.
9. Pipette.

2.2 Screening of Chitinase-Producing Bacteria

1. Nutrient agar (supplemented with sucrose/glucose).
2. Colloidal chitin.
3. 0.1% congo red stain.
4. 1% NaCl.
5. Laminar air flow.
6. Incubator (4 °C).
7. Wire loop.
8. Glass spreader.
9. Parafilm.

10. Scale.
11. Distilled water.
12. Sugar tubes.
13. Pipette.
14. Petri plates.

2.3 Enzyme Production in Shake Flasks

1. Production medium: 1.0% swollen chitin, 0.5% peptone, 0.5% yeast extract, 0.1% KH_2PO_4 , and 0.01% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (pH 9.0).
2. Shaker.
3. Conical flask.
4. Majoring cylinder.
5. Incubator.
6. Parafilm.
7. Distilled water.
8. Sugar tubes.
9. Pipette.
10. Spectrophotometer.

2.4 Purification of Chitinase Enzyme

1. 50 mM Tris-HCl buffer, pH 9.0.
2. Ammonium sulfate.
3. DEAE (Diethylaminoethyl)-cellulose column (2.0×25 cm).
4. NaCl (0–1 M in 100 mM Tris-HCl buffer).
5. Colloidal chitin.
6. Conical flask.
7. Centrifuge (4 °C).
8. 1.3% suspension of colloidal chitin in 50 mM Tris-HCl buffer (pH 9.0).
9. Incubator (37 °C).
10. Centrifuge.
11. Dinitrosalicylic acid (DNS).
12. *N*-acetylglucosamine.
13. Bovine serum albumin (BSA).
14. Spectrophotometer.
15. Test tubes.
16. Pipette.
17. (5 mM concentration).
18. Eppendorf tubes (1.5 ml).
19. Colloidal chitin.

2.5 Effects of Temperature, pH, and Various Metal Ions on Enzyme Activity and Stability

2.5.1 Enzyme Assay

1. 50 mM Tris-HCl buffer (pH 9.0).
2. Chitin.
3. Dinitrosalicylic acid (DNS).
4. *N*-acetylglucosamine.
5. Incubator.
6. Centrifuge.

2.5.2 Effect of Temperature for Enzyme Activity and Thermostability

1. Eppendorf tubes (1.5 ml).
2. 50 mM in Tris-HCl buffer (pH 9.0).
3. Colloidal chitin as a substrate.
4. Water bath.

2.5.3 Effect of pH on Enzyme Activity and Stability

1. Citric acid- Na_2HPO_4 (pH 5–6).
2. Phosphate buffer (pH 6–8).
3. Boric acid-NaOH buffer (pH 8–9.5).
4. Phosphate-NaOH buffer (pH 9.5–12).
5. Sodium hydroxide solution buffer (pH 12–13).

2.5.4 Effect of Metals on Enzyme Activity

1. Different metals (5 mM concentration).
2. MnSO_4 .
3. FeSO_4 .
4. CoCl_2 .
5. MgSO_4 .
6. CuSO_4 .
7. CaCl_2 .
8. EDTA.
9. HgCl_2 .
10. NiCl_2 .

2.6 Molecular Weight Determination by SDS-PAGE

1. 5% stacking gel and 12% resolving gel.
2. Standard molecular marker (6.5–200 kDa).

3 Method

3.1 Isolation and Identification of Chitinase-Producing Bacteria (See Note 1)

1. Isolate the chitinase-producing bacteria from marine sediment using an enrichment procedure.
2. Serially dilute and spread the enriched culture broth on chitin agar plates containing (g/l) 1.0% swollen chitin, 0.5% peptone, 0.5% yeast extract, 0.1% KH_2PO_4 , 0.01% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (pH 9.0) and incubate at 37 °C for 48 h.

3. Screen the isolated colonies for further chitinase production.
4. Consider the colonies with highest clear zone as potential strain and subject to morphological and biochemical identification.
5. Identify the chitinase-producing bacterial strain according to Bergey's Manual of Determinative Bacteriology [11].

3.2 Screening of Chitinase-Producing Bacteria [12]

1. Carry out the quadrant streak of all the isolates in nutrient agar plate supplemented with colloidal chitin to isolate the potential organism based on the chitinase produced.
2. Perform the single streak inoculation measuring 2 cm length for all the bacterial isolates on nutrient agar medium supplemented with colloidal chitin.
3. Incubate it at room temperature for 2 days.
4. Stain the plates with 0.1% congo red and destained with 1% NaCl.
5. Select the bacterial isolates producing a clear zone of more than 10 mm for the study.
6. Store the screened pure isolates in nutrient slants with 1% colloidal chitin at 4 °C to maintain the viability of chitinase producers.

3.3 Enzyme Production in Flasks

1. For production of chitinase enzyme, culture the bacteria into the production medium which contains 1.0% swollen chitin, 0.5% peptone, 0.5% yeast extract, 0.1% KH_2PO_4 , and 0.01% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (pH 9.0).
2. Seed the culture medium with 1% inoculum (1.85×10^6 CFU/ml) and maintain at above culture conditions (pH 9.0, temperature 37 °C, and 1.0% chitin and 150 rpm) for 48 h.
3. Determine the bacterial growth and enzyme production from the samples collected at 6 h intervals.
4. Estimate the growth by measuring optical density (O.D.) of the culture broth collected at different time intervals at 600 nm.

3.4 Purification of Chitinase Enzyme

1. Harvest the culture from the shake flask after 72 h and centrifuge at $6000 \times g$ for 30 min at 4 °C to remove the cells.
2. Use the cell free supernatant as crude enzyme.
3. Precipitate the crude chitinase with ammonium sulfate at 60% saturation and allow to stand overnight at 4 °C.
4. Collect the precipitates by centrifugation and dialyze against 50 mM Tris-HCl buffer, pH 9.0 for 24 h.
5. Load the dialyzed enzyme solution onto a DEAE (Diethylaminoethyl)-cellulose column (2.0×25 cm) and equilibrate with Tris-HCl buffer, pH 9.0.
6. Dilute the enzyme with linear gradient of NaCl (0–1 M in 100 mM Tris-HCl buffer) at a flow rate of 25 ml/h.

7. Assay the eluted fractions for enzyme activity.
8. Determine the amount of protein in crude as well as purified enzyme [13].
9. Use bovine serum albumin (BSA) as standard for protein estimation.
10. Use this partially purified enzyme solution to investigate the effects of temperature, pH, and various metal ions on enzyme activity and stability.

3.5 Effects of Temperature, pH, and Various Metal Ions on Enzyme Activity and Stability

3.5.1 Enzyme Assay

1. Measure the chitinase activity with colloidal chitin as a substrate.
2. Add the enzyme solution (0.5 ml) to 1.0 ml of substrate solution, which contain a 1.3% suspension of colloidal chitin in 50 mM Tris-HCl buffer (pH 9.0).
3. Incubate the mixture at 37 °C for 30 min.
4. After centrifugation, determine the amount of reducing sugar produced in the supernatant by the dinitrosalicylic acid (DNS) method [14] with *N*-acetylglucosamine as a reference compound.
5. One unit of chitinase activity is defined as the amount of enzyme that produces 1 mol of reducing sugars per min.

3.5.2 Effect of Temperature for Enzyme Activity and Thermostability

1. Incubate the reaction mixture at different temperature levels to determine the optimum temperature for enzyme activity.
2. Perform the thermostability of the enzyme in eppendorf tubes (1.5 ml) containing 0.2 ml of purified enzyme in 50 mM in Tris-HCl buffer (pH 9.0).
3. Incubate the purified enzyme at 35, 40, 50, 60, 70, 75, 80, 85, 90, and 95 °C for 1 h aliquots and withdrawn every 15 min
4. Determine the residual activity by standard assay condition with colloidal chitin as a substrate.

3.5.3 Effect of pH on Enzyme Activity and Stability

1. To check the effect of pH on enzyme activity, carry out with five different buffer systems (50 mM): Citric acid- Na_2HPO_4 (pH 5–6), phosphate buffer (pH 6–8), boric acid-NaOH buffer (pH 8–9.5), phosphate-NaOH buffer (pH 9.5–12), and sodium hydroxide solution buffer (12–13) and determine the residual activity by the standard assay.
2. Evaluate the stability of the enzyme by incubating the enzyme solution at different pH for 1 h and assay the residual activity.

3.5.4 Effect of Metals on Enzyme Activity

1. Determine the effect of metals on enzyme activity by incubating the enzyme with different metals (5 mM concentration) at room temperature for 1 h.
2. Consider the activity of enzyme without any metal as control and the activity was taken as 100%.

3.6 Molecular Weight Determination by SDS-PAGE (see Notes 2 and 3)

1. Perform the SDS-PAGE to estimate the molecular weight of the purified protein using 5% stacking gel and 12% resolving gel [15].
2. Estimate the molecular weight by comparing the relative mobility of proteins of different molecular sizes, using a standard molecular weight marker (205, 97.4, 66, 43, and 29 kDa).

4 Notes

1. Examples of chitinase-producing bacterial species are *Vibrio* sp. [16], (*Enterobacter* sp.). [17], *Aeromonas* sp. [18], and *Alcaligenes xylosoxydans* [19].
2. The molecular weight of bacterial chitinases are mostly around 60,000–110,000, while those of actinomycetes are mostly 30,000 or lower, fungi are higher than 30,000.
3. The molecular weight of plant chitinases are mostly around 30,000. The enzyme is highly active in the temperature range of 30–50 °C but optimum at 40 °C and pH 8.0.

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Chapter 41

Glucanase

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Abstract

Beta glucanase is an enzyme that hydrolyzes β -glucans. β -Glucanases are important in that they are needed to break down the complex β -glucan molecules into smaller units. There is a wide range of such enzymes, differing in the specific bonds that are broken. Enzymes excel in their ability to alter the chemical composition of the substrate. The enzymes of suitable purity and cost are used to achieve the desired chemical conversion without the formation of a deleterious side product, and this can be obtained at moderate temperature and neutral pH. Enzymatic preparations from natural extract or food materials are used as food additives but currently, animal, fungal, or even bacterial enzymes are also acceptable. Most of the natural products are biodegradable due to the action of enzymes present in environmental micro-organism. These natural products can be hydrolyzed by using enzymes such as hydrolases like glucanases or proteases, lipases, and nucleases. The expensive co-factors are not needed but it is necessary to choose the enzyme based on its substrate specificity.

Key words β -Glucanase, Hydrolytic enzyme, Biocontrol

1 Introduction

Polysaccharides metabolizing enzymes were used in baking and brewing processes since ancient times even before the relationship between the chemical structure of polysaccharides and their modification by enzymes was studied. The studies of the structure and function of polysaccharides and their role involved in the a) storage of chemical energy, b) structural parts of tissue as well as c) their turnover by the enzymes that metabolize them, have been carried out in great detail. The enzymes have been used as basic tools in the determination of the structure of polysaccharides. The specificity of enzymes towards anomeric linkage of sugar allows a true determination of this aspect. Enzymatic procedures used in the structural studies are rapid and can be used on a microscale. Studies on polysaccharides can be performed in a mixture but with the addition of a specific enzyme. An advantage of enzymatic hydrolysis over acidic hydrolysis is that much higher yields can be expected

with no artifacts. Again enzymatic hydrolysis occurs without the use of high temperatures and acidic or alkaline conditions. The optimal conditions required for enzymatic hydrolysis are near neutral pH values and temperatures near room temperatures. These conditions ensure that only selected bonds can break and sugars and conjugate structures are not altered by extremes of pH and high temperatures. Acidic or alkaline conditions which are used for the structural determinations of polysaccharides modify low molecular weight products and may mislead the conclusions. Complex polysaccharides can also be modified with the help of selective enzymes.

Among the many glucanases, α -glucanases are widely used in food processes industries. The α -glucanases of fungal origin have important applications in the brewing industry, where it can degrade the barley beta-glucans in the wort that is reduced to products having high viscosities causing pumping and filtering problems [1]. α -glucanases are also useful, in the degradation of wheat flour to improve baking quality [2].

Cellulose (β -1,4-D-glucan) and β -glucans (β -1,3(4)-D-glucan) are among the most abundant groups of naturally occurring polysaccharides [3]. Many microorganisms are capable of degrading cellulose and β -glucans by producing enzymes such as cellulases/ β -1,4-endoglucanase and β -glucanases/ β -1,3 (4)-endoglucanases. These enzymes are commercially produced for various industrial applications by the mass fermentation of high enzyme yielding microorganisms generated through mutation, screening, and selection. The cost associated with the development of high enzyme yielding microorganisms is dependent on the efficiency of the screening step. β -endoglucanase activities are determined by the extent to which the natural polysaccharide substrate is hydrolyzed. Microorganisms can be screened for β -endoglucanase production by measuring their ability to grow on defined or semi-defined media [4] by growing cultures in conjunction with an indicator of enzyme activity or by measuring the extracellular enzyme activity in spent fungal cultures. Growing cultures on specific media capable of enzyme detection are the simplest screening method, but it may take days to visualize enzyme activity. Alternatively, measuring cell-free enzyme activity in spent culture filtrates is more rapid, but it may also require more processing [5]. Development of defense responses in plants is complex. It involves both structural and biochemical barriers [6, 7]. Though structural barriers are significant, the synthesis of new proteins that have direct or indirect action on the course of pathogenesis has a major effect on plant resistance to a given pathogen. These proteins include a heterogeneous group of proteins collectively defined as pathogenesis-related (PR) proteins [8]. Among these PR proteins, hydrolases such as β -1,3-glucanases and chitinases have been suggested to be involved in plant resistance against fungal pathogens

[9–14]. These enzymes, developmentally regulated, are induced by pathogens or exogenous chemicals ([9, 15, 16], inhibit fungal growth [17] and release oligosaccharide elicitors inducing the production of phytoalexins [18, 19].

2 Materials

2.1 Screening of Glucanase (β -1,4-Endoglucanase) Producing Fungal Isolates by Microplate Diffusion Assay

2.1.1 Preparation of Assay Medium (See Note 1)

1. Microorganisms—fungal strain (*Trichoderma harzianum*, *Coniothyrium minitans*).

1. 0.2% AZCL-hydroxyethyl-cellulose.
2. 2% agar dissolve.
3. 25 mM sodium acetate.
4. pH meter.
5. Autoclave.

2.1.2 Procedure for Microplate Diffusion Assay

1. Pipette.
2. 96-well microplate.
3. Nutrient agar medium.
4. Cheesecloth.
5. β -1,4-endoglucanase (Megazyme).
6. 25 mM sodium acetate (pH 4.5).
7. Test fungal culture.
8. 25 mM sodium acetate (pH 4.5).
9. Incubator (20–22 °C).
10. Spectrophotometer.
11. pH meter.

2.1.3 Detection of Glucanase Activity

1. 0.1 M sodium acetate buffer (pH 5.0).
2. 1% (w/v) laminarin.
3. 0.8% (w/v) agarose.
4. Nutrient agar (supplemented with sucrose/glucose).
5. 0.1% congo red stain.
6. Laminar air flow.
7. GS-800 calibrated densitometer.
8. Incubator.
9. Wire loop.

10. Glass spreader.
11. Parafilm.
12. Distilled water.
13. Sugar tubes.
14. Pipette.
15. Petri plates.

2.2 Enzyme Production and Assay

1. Potato-dextrose agar.
2. TLE liquid medium (0.1% bactopectone; 0.03% urea; 0.2% KH_2PO_4 ; 0.14% NH_4SO_4 ; 0.03% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$; 0.03% $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$; 1 mL of 0.01% trace elements solution such as Fe^{2+} , Mn^{2+} , Zn^{2+} , and Co^{2+} , 0.02% glucose, pH 5.5).
3. Laminarin solution (1%).
4. Sodium acetate buffer, pH 5.0.
5. 3,5-dinitrosalicylic acid reagent.
6. Incubator shaker.
7. Petri plates.
8. Conical flask.
9. Distilled water.
10. Sugar tubes.
11. Whatman filter No.1.
12. Laminarin solution (1%).
13. pH meter.
14. 50 mM sodium acetate buffer, pH 5.0.
15. 3,5-dinitrosalicylic acid reagent.
16. Spectrophotometer.
17. sodium acetate (pH 3.0–5.0) and (pH 6.0–8.0).
18. Tris–HCl (pH 9.0).

2.3 Enzyme Purification

1. Incubator (4 °C).
2. Centrifuge machine.
3. Ammonium sulfate.
4. Sodium acetate pH 5.0.
5. Phenyl Sepharose CL 4B column (10 × 1.8 cm).
6. Acetate buffer, pH 5.0.
7. Distilled water.
8. Electrophoresis gel instrument.
9. Silver reagent.

3 Methods

3.1 Screening of β -1,4-Endoglucanase Producing Fungal Isolates by Microplate Diffusion Assay (See Note 2)

3.1.1 Preparation of Assay Medium

1. Take 0.2% AZCL-hydroxyethyl-cellulose and 2% agar dissolve in 25 mM sodium acetate, and adjust pH to 4.5.
2. Then autoclave the assay medium and cool to 55 °C.

3.1.2 Procedure for Microplate Diffusion Assay (See Note 5)

1. 20 mL of the assay medium will be pipetted out into the lid of a 96-well microplate.
2. Once the medium is solidified, cut to fit a single layer of sterile cheesecloth for 96-well microplate lid and place carefully on the assay medium, thereby lining the lid and reinforcing the assay matrix.
3. Dilute standard β -1,4-endoglucanase (Megazyme) in 25 mM sodium acetate (pH 4.5) to final concentrations of 4, 2, 1, 0.75, 0.5, 0.25, and 0.00 U/mL, add 50 μ L each and prepare standard sample into a designated row of wells in the 96-well microplate diffusion assay (in duplicate).
4. Then add 50 μ L each test fungal culture supernatant into a designated well and repeat in triplicate.
5. All the sample wells in the diffusion assay plate are subsequently filled with 25 mM sodium acetate (pH 4.5) to a final volume of 350 μ L.
6. The lid line with the assay medium is placed on top of the sample-fill, 96-well microplate assay and invert while firmly pressing the lid and plate together.
7. To ensure direct contact between enzyme sample and the assay medium, remove all air bubbles by firmly pressing the bottom of the plate (forcing any air bubbles to rise to the top of the well).
8. Incubate the assay plates for several hours at room temperature (20–22 °C).
9. To measure the enzyme activity, the plates are flip upright, remove the lids, and the absorbances of samples will be read on an automatic microplate reader at 590 nm.

3.1.3 Detection of Glucanase Activity

1. Screen the selected microorganisms on agar plates consisting of 0.1 M sodium acetate buffer (pH 5.0), 1% (w/v) laminarin, and 0.8% (w/v) agarose.
2. Incubate the plates for 48 h at 37 °C.
3. Afterward stain the plates with 0.5 mg/L congo red [20].

4. Clear zones in a red background indicate the glucanase activity.
5. Digitalize the congo red-stained plates using a GS-800 calibrated densitometer.

3.2 Enzyme Production and Assay

1. For enzyme production, *T. harzianum* spores (2×10^6) inoculate in 250 mL TLE liquid medium (0.1% bactopectone; 0.03% urea; 0.2% KH_2PO_4 ; 0.14% $(\text{NH}_4)_2\text{SO}_4$; 0.03% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$; 0.03% $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$; 1 mL of 0.01% trace elements solution (Fe^{2+} , Mn^{2+} , Zn^{2+} , and Co^{2+}), 0.02% glucose, pH 5.5) containing chitin (0.5%), for 72 h at 28 °C. The cultures incubate for 0–72 h at 28 °C, with agitation (250 rpm).
2. The culture supernatants separate from the mycelium by filtration through filter paper, and use for enzyme assay and/or enzyme purification as described in the section below.
3. β -glucanase activity assay (in triplicate) using a reaction system containing 250 μL of a laminarin solution (1%) dissolve in 50 mM sodium acetate buffer, pH 5.0 and 0–125 μL of enzyme solution. Reaction allow to process for 30 min at 37 °C and stop by addition of 1.5 mL of 3,5-dinitrosalicylic acid reagent.
4. The reducing sugar form is then determined spectrophotometrically at 550 nm [21]. One unit of enzyme (U) is defined as the amount of protein necessary to produce one micromole of reducing sugar per min (See **Notes 3** and **4**).
5. For determination of optimum pH, the assays run for 30 min at 37 °C, using the buffer solutions (50 mM) of sodium acetate (pH 3.0–5.0), sodium phosphate (pH 6.0–8.0), and Tris–HCl (pH 9.0) containing the substrate laminarin. In all cases, the standard deviation values for enzyme activity were smaller than 5% of the mean values. For determination of KM and V_{max} of laminarin hydrolysis, the concentrations of this substrate varied from 0.1 mg to 20.0 mg/mL.

3.3 Enzyme Purification

1. To the supernatants from *T. harzianum* cultures obtained as described above, $(\text{NH}_4)_2\text{SO}_4$ is added to a concentration of 40%.
2. The mixtures are kept for 15 min at 4 °C under gentle agitation. Centrifuge suspensions at $28,800 \times g$ for 30 min, and discard the pellets. Then add ammonium sulfate to supernatants for a final concentration of 60%.
3. After 15 min at 4 °C under gentle agitation, the supernatants centrifuged at $28,800 \times g$ for 30 min, and the resulting pellets resuspended in a small volume of 50 mM sodium acetate pH 5.0, and use as crude enzyme preparation.
4. Samples of crude enzyme load on a Phenyl Sepharose CL 4B column (10×1.8 cm) equilibrated with acetate buffer 50 mM, pH 5.0.

5. Proteins eluted with a negative gradient formed with 40 mL of the acetate buffer containing 1 M ammonium sulfate, and 40 mL of the acetate buffer. Collect the samples of 2.6 mL at a flow rate of 52 mL/h. Monitor protein ($A_{280\text{nm}}$) and β -glucanase activity for each sample. Pool the fractions containing enzyme, dialyze against distilled water, and concentrate by freeze-drying.
6. The concentration of protein in the enzyme samples is determined by a simplification of the method of Lowry et al. [22].
7. β -glucanase samples are analyzed by electrophoresis in polyacrylamide gels under denaturing conditions [23]. Proteins present in the gels stain with silver reagent [24].

4 Notes

1. The AZCL-hydroxyethyl-cellulose is insoluble in buffered solutions; however, rapid hydration of the substrate by the assay medium will form a soluble gel particle available for β -1,4-endoglucanase digestion.
2. Digestion by β -1,4-endoglucanase releases blue dye fragments into the media, resulting in a visible blue color. Consequently, the blue dye diffuses from the solid agar assay medium and into the contacting sample/enzyme solution within the wells of a 96-well microplate.
3. As a result, a more intense blue color will develop in the sample well containing relatively higher β -1,4-endoglucanase activity.
4. Sample activity will be predicted from a standard curve produced from the relative absorbances of the standard β -1,4-endoglucanase samples in each individual plate.
5. To minimize the diffusion of dye and enzyme between sample wells, the enzyme reaction will be terminated by removing the lid as soon as the blue color became visible in the wells of the assay plate.

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Identification of Cellulase Enzyme Involved in Biocontrol Activity

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Abstract

Biocontrol agents produce lytic enzymes and cellulase is one of them to limit invasion of pathogen to the plants. Application of biocontrol agent in agricultural industry has enabled researchers to investigate cellulase enzyme activity. Bacterial source majorly challenges fungi phytopathogens. Apart from biocontrol, cellulase has plant growth promoting traits. Here, qualitative and quantitative methods are provided to assay cellulase from potential isolated biocontrol agent. To determine antagonistic efficacy to pathogens of biocontrol agent under field conditions, it is required to evaluate the agent using different identifier and cellulase production is one of them.

Key words Cellulase, Cellulose, DNS, CMC, Endoglucanase

1 Introduction

Cellulase is an enzyme which hydrolyzes cellulosic compound. It belongs to the glycoside hydrolase family that catalyze the glycosidic bond hydrolysis [1] and hydrolase superfamily that utilize water molecule to break large molecule. Three main categories of cellulases based on their mode of action include (1) endoglucanase, which targets β -1,4 glycoside linkages randomly; (2) exoglucanase, which targets β -1,4 glycosidic bond either from non-reducing end or reducing end of polymer; (3) β -Glucosidase, which targets non-reducing end and liberate glucose molecule. Cellulase enzyme is produced extracellularly by various microorganisms during their growth phase [2]. *Bacillus*, *Cellulomonas*, *Pseudomonas*, *Clostridium*, *Erwinia*, *Fibrobacter*, *Ruminococcus*, *Butyrivibrio*, *Bacteroides* [3, 4], and *Streptomyces* [5] are the bacterial genera while *Aspergillus*, *Rhizopus*, *Trichoderma*, *Fusarium*, *Neurospora*, and *Penicillium* are fungi genera and actinomycetes including *Hermomonospora*, *Hermoactinomyces* [3] are known to produce cellulases. Cellulases are known to involve in biocontrol of pathogenic microorganism to

plants and it is required to evaluate an isolate on the basis of different parameters; cellulase is one of them. Biocontrol bacterial cellulase targets cellulose in the cell wall of fungi which are pathogenic to plants [6]. *Bacillus* spp. produces cellulase described to act as plant growth promoter [7]. However, on other side, plant pathogen produces enzyme to invade plant cell wall and cellulase is one of them [8, 9]. Positive correlation between enzyme production, virulence, and disease symptoms has been established [10].

This chapter provides methodology to check presence or absence and/or amount of cellulase enzyme in potential isolates. Carboxymethyl cellulase (CMCase) activity assay [11] is a qualitative method when presence or absence of enzyme checked, it becomes semi-quantitative when diameters of zone of clearance of isolates are compared to identify higher enzyme producer. Cellulase enzyme activity assay [11, 12] is a quantitative method which indirectly determines the level of enzyme based on generation of colorimetric product. Both assays are measuring endoglucanase which represents cellulase unless specific cellulase enzyme type targeted. Error source and assay operation may affect the sensitivity of both assays.

2 Materials

Use ultrapure water and analytical grade reagent while preparing the solutions and media. Prepare media as per manufacturer's instruction and store at room temperature unless indicated; same follows for solutions. Follow appropriate good laboratory practices for disposal of waste materials.

2.1 CMCase Activity

1. CMC agar: 0.4 g/l KH_2PO_4 , 0.02 g/l CaCl_2 , 0.02 g/l NaCl , 0.02 g/l $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 2.5 g/l CMC, 15.0 g/l agar. Adjust pH to 7.2 with 1 M NaOH .
2. Congo red solution: Dissolve 1 mg/ml solution in ultrapure water.
3. Sodium chloride solution: 1 M solution in ultrapure water.

2.2 Cellulase Enzyme Activity Assay

1. Cellulase production medium: 0.25% (w/v) yeast extract, 0.5% K_2HPO_4 , 0.1% NaCl , 0.02% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.06% $(\text{NH}_4)_2\text{SO}_4$, and 2.0% carboxymethyl cellulose (CMC) as carbon source. Adjust pH to 7.0 with 1 M NaOH .
2. 3,5-dinitro salicylic acid (DNS) reagent: (a) 10 g DNS in 200 ml 2 M NaOH ; (b) 300 g sodium potassium tartrate in 500 ml ultrapure water. Prepare DNS solution by mixing solution (a) and (b), and make up volume to 1 liter with ultrapure water just before use.

3. Glucose standards: Prepare 100 ml 2 mg/ml solution of glucose. Prepare known glucose standards by diluting 2 mg/ml glucose solution in ultrapure water. For example, 0.2 ml, 0.4 ml, 0.6 ml, 0.8 ml, and 1.0 ml of 2 mg/ml of glucose solution in 0.8 ml, 0.6 ml, 0.4 ml, 0.2 ml, and 0.0 ml of ultrapure water, respectively, totalling 1 ml of each dilution.
4. Citrate buffer: Prepare 50 mM citrate buffer in ultrapure water and set pH 4.8 with 1.0 M NaOH.
5. CMC substrate: Add 2.0 g of CMC (2% w/v) per 100 ml of 50 mM citrate buffer. Dissolve the CMC and heat the solution if required.

3 Methods

3.1 Carboxymethyl Cellulase (CMCase) Activity (see Notes 1–4)

1. Inoculate loopful culture of an isolate to Luria Bertani growth media and incubate at 37 °C temperature at static or shaking condition overnight.
2. Re-inoculate overnight grown culture of an isolate to new fresh sterile Luria Bertani growth media and incubate at 37 °C temperature under shaking condition (120 rpm) till 0.6 OD against blank.
3. Spot the loopful of culture of 0.6 OD on CMC agar plates.
4. Incubate inoculated CMC agar plates at 37 °C temperature for 10 days in inverted position.
5. At the end of incubation, flood the plate with Congo red solution (1 mg/ml) for 15 min at room temperature.
6. Pour off the Congo red solution and flood again the plate with 1 M NaCl for 15 min at room temperature.
7. Zone of clearance shows cellulase activity on CMC plate. Measure the diameter of clear zone around the colony as well as also measure diameter of the colony.
8. Calculate the ratio of the clear zone diameter to colony diameter (Fig. 1). Largest the ratio higher the cellulase producing isolate and vice-versa.

3.2 Cellulase Enzyme Activity Assay (see Notes 5–8)

1. Cellulase production: Inoculate 1 ml or 1 O.D. culture of 0.6 O.D. in 250 ml Erlenmeyer flasks containing sterile 100 ml of cellulase production medium and incubate at 37 °C on rotatory shaker at 120 rpm under aerobic condition.
2. Crude enzyme preparation: Withdraw 1 ml of sample at intervals and centrifuge for 10,000 rpm ($11,200 \times g$) for 10 min at 4 °C. Collect the supernatant which is source of crude enzyme. Store the crude enzyme at 4 °C until next processing.

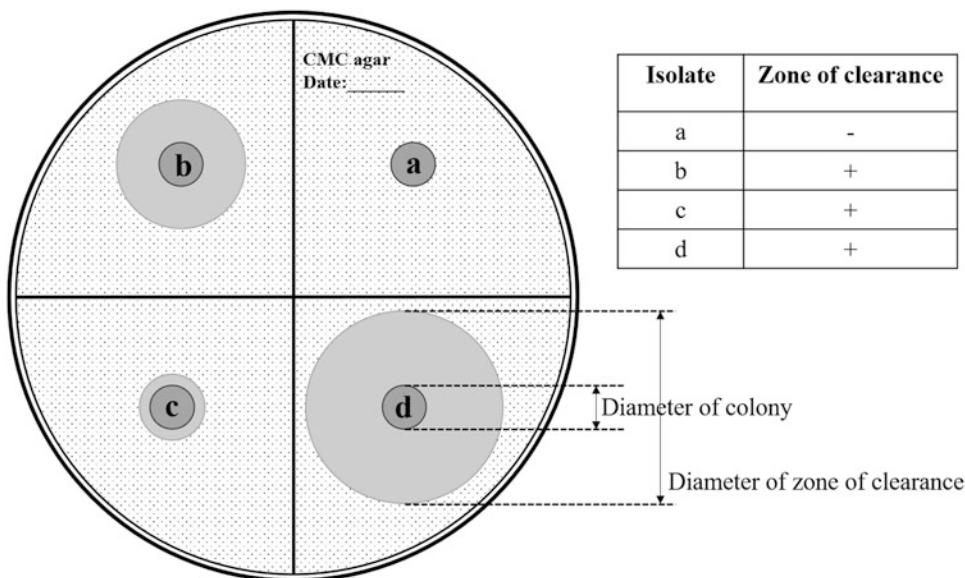


Fig. 1 CMC agar plate inoculated with four isolates

3. Standard glucose curve: Mix 1.0 ml of each dilution and blank (ultrapure water) to 3.0 ml of DNS solution and follow **Step 6** onwards.
4. Mix 0.5 ml of CMC substrate to 0.5 ml crude enzyme (or appropriate dilutions) and incubate at 50 °C for 30 min.
5. Stop the reaction by adding 3.0 ml DNS reagent and mix.
6. Incubate for 5 min in boiling water-bath and cool the reaction under tap water.
7. Make up total volume 10 ml using ultrapure water and mix.
8. Prepare enzyme blank (without CMC substrate) and substrate blank (without enzyme) and follow from **Step 5 to 7**.
9. Read absorbance of all tubes at 540 nm.
10. Prepare standard curve of glucose plotting concentration on γ -axis and O.D. on X -axis (*see Note 9*).
11. Subtract enzyme blank O.D. from substrate blank O.D. and find amount of reducing sugar released by enzyme using standard glucose curve.
12. Determine enzyme activity considering one unit of enzyme which is defined as one micromole of glucose liberated per min under standard assay conditions.

4 Notes

1. Aseptic condition is a good laboratory practice used for microbial study that includes isolation, culture growth, and culture transfer. Horizontal laminar air flow should be used for microbial culture work using sterile requirements.
2. Autoclave liquid as well as solid media used for microbial culture at 121 °C at 15 psi pressure for 20 min.
3. Incubation temperature, growth medium, and incubation time for plate in CMCase assay may vary according to the isolate type.
4. Alternate to spot of an isolate on CMC agar plate, well of 4–10 mm diameter using cork borer is also recommended.
5. Filter the DNS solution using Whatman filter paper and store in amber bottle at room temperature. It can be stored for at least 24 months.
6. For cellulase enzyme activity assay, cellulase production medium composition may vary according to isolate type.
7. If the concentration of reducing sugar in the mixture is high, the crude enzyme may need to be diluted further in 50 mM citrate buffer. Use the dilution factor while calculating the enzyme activity.
8. Prefer monohydrate for preparation of citrate buffer.
9. For standard glucose curve, the preparation of glucose concentrations and volumes can be varied as appropriate.

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Identification of Protease Enzymes Involved in Biocontrol Activity

Vimalkumar Prajapati, Swati Patel, Sanket Ray, and Kamlesh C. Patel

Abstract

Many microorganisms have plant growth-promoting activity and biocontrol activity, which is mainly imparted by their metabolic potential to the produced hydrolytic enzyme, such as proteases. The hydrolytic enzymes, especially alkalophilic proteases produced by many bacteria, fungi, and actinomycetes can be used as potential biocontrol agent against many plant pathogens. Proteases found to be resistant to extreme conditions and are showing great ability to inhibit and eradicate detrimental parasite, which create intensity to improve their performance in field conditions. Protease production can be accessed qualitatively and quantitatively, employing numerous methodology. Skim Milk Agar (SMA) is a widely adopted method for initial screening but the modified procedure of SMA is more advantageous than the conventional as it can be helpful in the precise determination of proteases by slow growing and fast-growing fungi with fairness.

Key words Extracellular proteases, Biocontrol agent, Enzymatic potential, Antagonistic, Skim milk agar (SMA)

1 Introduction

Biological control or biocontrol is generally defined as **controlling pests** such as **insects**, **mites**, **weeds**, and plant diseases using other organisms. Biological control agents of plant diseases are most often referred to as antagonists. Numerous bacterial, actinomycetes, and fungal species have been identified and used as biocontrol agents. Many *Bacillus* species have been exploited for their important metabolite, which contributes to potential biocontrol activity. More specifically, *Bacillus thuringiensis*, a soil-dwelling bacterium, is the most widely applied species of bacteria as biocontrol. Several fungi, e.g., *Beauveria bassiana*, *Lecanicillium* spp., *Metarhizium* spp., *Paecilomyces fumosoroseus*, *Purpureocillium lilacinus*, *Trichoderma* species, *Cordyceps*, *Metacordyceps*, Chytridiomycota, and Blastocladiomycota, have been exploited for their biocontrol potential.

Among all the plant parasite, root-knot nematodes (RKNs) are responsible for causing approximately half of total damages to the crops. Because of the infective capabilities [1], RKNs as *Meloidogyne incognita* incursions resulted in losses equal to \$400 million per year [2]. Various chemical and pesticide are used to control the pathogens and pests, but those chemicals lead to ecological hazards and are toxic to the human [3]. The green alternative to overcome these problems is biological control using microorganisms with potential active metabolites against all kind of plant pathogens [4]. The biocontrol potential of all the microorganisms is mainly because of their certain metabolite production, specifically the hydrolytic enzyme, i.e., proteases, chitinases, etc. which established them as suitable biocontrol agents against many plant pathogens. *Trichoderma* spp. and *Bacillus* spp. have been explored long back for its antagonistic and biocontrol activities which is mainly attributed by its extracellular protease enzyme production [4]. Proteases are broadly classified into two major types, viz., exoproteases (EC 3.4.1.1-19) and endoproteases (EC 3.4.2.1-24). They are further classified as acid proteases, cysteine proteases, serine proteases, and metalloprotease based on the amino acid at the active site. Protease is one of the major industrial enzymes, which accounts for ~50% of the world industrial enzyme market.

2 Materials

2.1 Qualitative Detection of Proteases

2.1.1 Skim Milk Agar Plate Assay

1. Distilled water.
2. Skim milk.
3. Mycological peptone/bacteriological peptone.
4. NaCl.
5. Agar powder.
6. Glass Petri plate.
7. Nichrome wire loop.
8. Erlenmeyer flasks.
9. Cup borer.
10. Incubator.
11. Autoclave.
12. pH meter.

2.1.2 Splitting-SMA (SSMA) Method

1. Potatoes.
2. Dextrose.
3. Cetyltrimethylammonium bromide (CTAB).
4. Other mentioned as above.

**2.1.3 Supplemented
Minimal Medium with Skim
Milk Agar (MSMW) Method**

1. MgSO_4 .
2. $(\text{NH}_4)_2\text{SO}_4$.
3. KH_2PO_4 .
4. Citric acid monohydrate.
5. Bromocresol purple.
6. Tween-80.
7. KOH.
8. Other mentioned as above.

**2.2 Quantitative
Detection of Proteases**

1. 0.5% casein solution.
2. 0.2 M Tris-HCl buffer of pH 8.0.
3. Trichloroacetic acid (110 mM).
4. 500 mM Na_2CO_3 .
5. Folin-Ciocalteu's reagent.
6. Tyrosine.
7. TCA.
8. Spectrophotometer.
9. Cooling centrifuge.
10. Test tubes.
11. Micro pipette and tips.
12. Crude/partially purified/purified enzyme solution.

**2.3 Assessment
of the Antagonistic
Activity of Cell-Free
Supernatant or
Purified Protease**

1. Malt extract agar (MEA).
2. *Fusarium oxysporum* (representative plant pathogen fungus).
3. Other mentioned as above.

3 Methods

**3.1 Qualitative
Detection of Proteases
(See Notes 1–3)**

**3.1.1 Skim Milk Agar
Plate Assay [5]**

1. The qualitative assessment of the protease production (fungi, bacteria, and actinomycetes) is carried out using skim milk agar (SMA) medium (1000 ml distilled water: 4 g skim milk, 1 g mycological peptone/bacteriological peptone, 5 g NaCl, 15 g agar) [see **Notes 1–3** for detail media composition].
2. Prepare skim milk agar and spot inoculate the respective biocontrol agent (bacteria and actinomycetes are spot inoculated while fungi as a disk of 5 mm diameter).
3. Incubate the inoculated plate at room temperature for 2–3 days and observe the clearance zone (halo) around colonies on SMA. The radius of the halo is used as a proxy of the production of proteases.

3.1.2 *Splitted-SMA (SSMA) Method* [5]

1. To perform this method, use two different media, including SMA and home-made potato dextrose agar (H-PDA, 1000 ml sterile distilled water, 200 g potatoes, 20 g dextrose).
2. Prepare the above media, pour into Petri dishes and allow to solidify.
3. Mark the solidified medium into two halves. Replace one part with SMA medium (50 °C) supplemented with cetyltrimethylammonium bromide (SMA-CTAB, 200 mg/L).
4. Streak inoculate the slow growing fungi on PDA part, while fast-growing fungi inoculate as disks (5 mm diameter in size).
5. Incubate plates at 25 °C in darkness.
6. The presence of zone of clearance indicates protease production on the SMA part of the split SMA plate. Measure the fungal growth covering H-PDA part of the split SMA plate when reaching to the borderline between H-PDA and SMA.
7. Measure the diameter of the clearance zone (halo) on SMA medium for each strain after 5 days.

3.1.3 *Supplemented Minimal Medium with Skim Milk Agar (MSMW) Method* [5]

This method is based on the supplementation of pH indicator dye in the medium. The protease production is ascertained based on the difference in diameter of violet halo formed on media plate.

1. Prepare minimal medium containing 0.3 g MgSO_4 , 3.0 g $(\text{NH}_4)_2\text{SO}_4$, 2.0 g KH_2PO_4 , 1.0 g citric acid monohydrate, 15 g agar, 0.15 g bromocresol purple, and 200 μl of tween-80 per liter in two different Erlenmeyer flasks.
2. Add skim milk (4 g/L) (MSMW) in one flask while another without SM (MSMWo).
3. Adjust the pH of the media to 4.7 with KOH.
4. After solidification of the medium, inoculate 5 mm disk diameter of respective fungal strains in the center of each Petri plate.
5. Incubate the Petri dishes at 25 °C for 5 days.
6. The difference in diameter of the purple halo formed by each of the strains on Petri dishes with and without skim milk is considered the amount of protease production.

3.2 *Quantitative Detection of Proteases*

3.2.1 *Determination of Proteolytic Activity* [6]

1. Use cell-free supernatant for the assessment of protease activity.
2. Prepare a reaction mixture consisting of 1 ml of enzyme, in a 5 ml of 0.5% casein solution in 0.2 M Tris-HCl buffer of pH 8.0.
3. Incubate the reaction mixture for 5 min at 60 °C.
4. Terminate the reaction by adding 5 ml of trichloroacetic acid (110 mM) and keep it for 30 min at room temperature and then centrifuge for 5 min at $11,200 \times g$.

5. After centrifugation, mix 2 ml of filtrate with 5 ml of 500 mM Na_2CO_3 solution followed by addition of 1 ml Folin–Ciocalteu’s reagent.
6. The amount of tyrosine released is determined spectrophotometrically at 660 nm against the enzyme blank.
7. The control is treated in the same way, except TCA is added before addition of enzyme solution.
8. One unit of protease activity is equivalent to the amount of enzyme that is required for releasing 1 μg of tyrosine/ml/min under standard assay conditions.

3.2.2 Protein Determination (See Note 4)

3.3 Antagonistic Activity of Cell-Free Supernatant or Purified Protease Against *Fusarium oxysporum* (Representative Plant Pathogen Fungus) [6]

1. Estimate the protein concentration of the culture filtrate using Lowry’s method [7].
1. In order to test the efficacy of crude or purified protease for antagonistic activity, add 100 μl of crude or purified protease into the well a little below the center of the pre-poured Petri plates of malt extract agar (MEA).
2. Place a mycelial disk from a 4-day old culture of *F. oxysporum* simultaneously on one side of the streak.
3. Inoculate pathogen only in the control plate for comparison.
4. Incubate the plates at $24 \pm 1^\circ\text{C}$, and calculate the percent growth inhibition according to Vincent (1947) [8].

$$I = (C - T/C) \times 100$$

where I = percent growth inhibition; C = growth of fungus in control; T = growth of fungus in treatment.

4 Notes

1. **Culture medium for the cultivation of *Actinomycetes* for the protease production as biocontrol agent [2]: Composition:** Glucose, 1%; Yeast extract, 0.5%; Peptone, 0.25%; Casein, 0.25%; MgSO_4 , 0.03%; FeSO_4 , 0.002%; ZnSO_4 , 0.02%; CaCO_3 , 0.1%; KH_2PO_4 , 0.1%; K_2HPO_4 , 0.1, pH 8.5; **Cultivation process:** Inoculate media with required density of organisms and incubate at 55°C for 96 h at 200 rpm shaking conditions. After incubation, cells are separated by centrifugation ($1789 \times g$, 10 min, 4°C) and the enzymes cell-free extracts are collected.
2. **Culture media for the cultivation of Bacteria for the protease production as biocontrol agent [2]: Composition:** (g/100 ml) casein, 2 g; yeast extract, 0.4 g; maltose, 2 g; KH_2PO_4 , 0.1 g; gelatin, 2 g. The pH of the medium was adjusted to pH 8.0 with 1 N NaOH for the production of

alkaline proteases; **Cultivation process:** Medium is inoculated with 1% (v/v) of 24 h old grown culture and incubated at 37 °C for 48 h on a rotary shaker at 100 rpm. The culture broth is then centrifuged at $16,128 \times g$ for 15 min after completing the incubation period. The obtained supernatant/clear filtrate is used as enzyme source.

3. **Culture media for the cultivation of Fungi for the protease production as biocontrol agent [9]: Composition:** Mineral medium containing (g/L): $MgSO_4$, 0.52; KCl, 0.52; KH_2PO_4 , 1.52; $FeSO_4 \cdot 7H_2O$, 0.01; $ZnSO_4 \cdot 7H_2O$, 0.01; and casein, 5 is used for the production of protease by fungal culture; **Cultivation process:** The production medium is inoculated with a 5 mm mycelial plug followed by incubation at 25 ± 2 °C for 7 days. The culture is then centrifuged ($2,800 \times g$, 10 min, 4 °C), and the supernatant (crude enzyme) is collected and used as enzyme source.
4. **Generalized protocol for Protease purification:** Enzyme production is carried out according to the process parameters as per the selection of the suitable biocontrol agents. Supernatant/filtrate is collected after centrifugation and subjected to precipitation technique using either of salt precipitation (ammonium sulfate) or solvent precipitation technique (acetone). Chilled acetone is added dropwise to the filtrate under continuous stirring in presence of ice bath, while ammonium sulfate is added pinch by pinch to the filtrate. The solutions is allowed to left in refrigerator overnight to enable the protein precipitation. The precipitated crude enzyme is then separated by centrifugation under cooling centrifuge at $5000 \times g$ for 10 min and dissolve phosphate buffer (pH 8.5) followed by dialysis. More purity is likely to be achieved after employing the various chromatographic technique, i.e., gel filtration chromatography (Sephadex G-100 column) and cation exchange chromatography [10].

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Isolation and Screening of Naringinase Producing Microbes: As Industrial Inputs for Agro Waste Base Enzyme Industry

Satish V. Patil, Jitendra D. Salunkhe, and Vishal Marathe

Abstract

Naringinase is an industrially important enzyme having wide applications in pharma and food industry. The enzyme is having a wide range of substrate, i.e., all natural glycosides containing α -L-rhamnose and β -D-glucose including quercitrin, rutin, naringin, hesperidin diosmin, etc. The enzyme majorly used for hydrolysis of glycosides. They are majorly used for biotransformation of steroid and antibiotics. Peels of all citrus fruits such as lime, lemon, grape fruit, and oranges are the major source of substrates of naringinase. This makes naringinase more attractive for cheap and ecofriendly production. The enzyme produced by various microorganisms like, fungi, bacteria, yeast, and actinomycetes.

The screening of potent enzyme producer with various qualities like alkaliphilic, acidophilic, and thermophilic is the need of current food industry. The proposed method of isolation and screening is based on selective medium for naringinase producer and detection by exposure to iodine vapor. The potent producer is screened by zone of clearance on purple brown background.

Key words Naringinase, Rhamnosidase, Glycosidase, Bitterness, Citrus peels

1 Introduction

Citrus fruits industry is one of the huge and important fruit industries. Besides the use of citric acid in food industry as acidulant, beverages and food preservative, its content are widely applicable in medicinal purposes such as kidney stone, gall action and anti-genotoxic effects [1].

Out of global production of citrus fruit, 34% was used for juice industry leading to production of about 44% peel waste [2]. Hence large amount of waste is produced. This citrus peel waste is the significant source of, pectin, limonene and remaining as cattle feed [3]. These peels also contain well-known flavanone glycoside “Naringin,” which is a major component contributing for bitterness in citrus juice. The debittering of citrus juice is managed by one of

commercially important enzyme known as naringinase which is the hydrolytic enzyme also known as α -rhamnopyranosidase. As per enzyme classification system it is known as L-rhamnosidase (E.C. 3.2.1.40) and β -D-glucosidase (3.2.1.21). The naringinase is not only important for juice industry as debittering agent but also yields commercially important products from citrus waste such as prunin.

Naringinase is also important in various pharmaceutical processes like steroid biotransformation, deglycosylation of glycopeptide antibiotics, flavonoids, or glycolipids [4]. Naringinase also uses various substrates as naringin, hesperidin, ter-phenyl glycosides, rutin, quercitrin, diosmin, as well as terminal α -rhamnose and β -glucose are the substrates of naringinase. Besides these naringinase has high commercial value due to its pharmaceutical properties as anticancer, anti-inflammatory, promotion of cell apoptosis in breast cancer, neuronal diseases like Epilepsy Alzheimer's disease and Parkinson's disease. Many microorganisms were recorded for naringinase production potential such as fungi and bacteria, e.g., *Aspergillus niger*, *A. oryzae*, *A. usamii*, *Burkholderia cenocepacia*, *Clostridium stercoarium*, and *Lactobacillus acidophilus*. Still there is urgent need of screening new potent producer microorganism which will be commercially important for food and pharmaceutical industry.

Principle: In the minimal medium, microorganism will produce naringinase enzyme for their energy need and will hydrolyze naringin producing zone of clearance or solubilization. Further confirmation could be also done by iodine overlay method in which iodine forms triiodine complex with unhydrolyzed naringin and gives purple brown color and remain colorless with hydrolyzed products which leads to formation of zone of clearance around colony (Fig. 44.1).

2 Materials

1. Naringin, Naringenin ($\geq 90\%$ from citrus fruit).
2. Iodine crystal.
3. Naringinase screening agar (g/l), $K_2HPO_4 \cdot 7.0$, $KH_2PO_4 \cdot 2.0$, Sodium Citrate 0.50, $MgSO_4 \cdot 0.10$, $(Na_4)_2SO_4 \cdot 1.0$, supplemented with 0.1% (w/v) naringin (Sigma-Aldrich) as sole carbon source (pH 7.0 ± 0.2) (see **Note 1**).
4. 4 M aqueous NaOH.
5. 0.1 M sodium acetate buffer (pH 4.0).

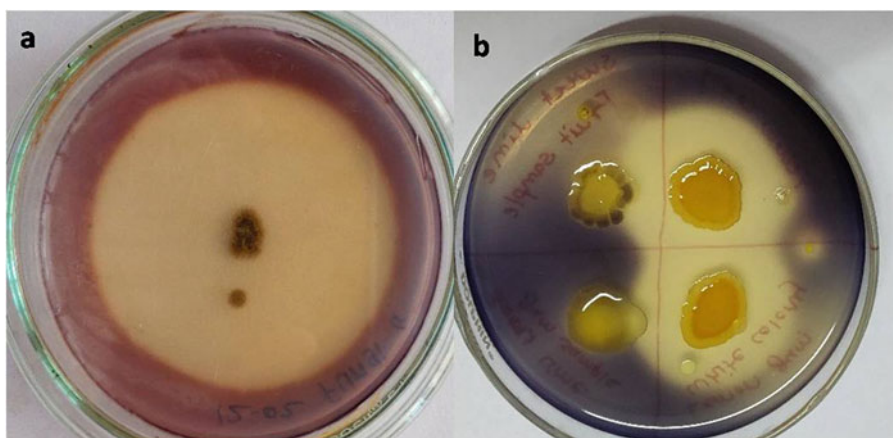


Fig. 44.1 Naringinase production confirmation by iodine vapors overlay method [4]. (a) Fungal isolate and (b) bacterial isolate showing zone of clearance (Source: Dr. Satish V Patil's, Labrotory, School of Life sciences, KBC NMU, Jalgaon)

3 Methods

1. Take 1.0 g of collected soil sample, add in 9.0 ml sterile saline, mix well by shaking and then further dilute up to ten folds, i.e., 10^{-9} .
2. Prepare minimal agar using pure distilled water in clean 250 ml Erlenmeyer flasks and sterilize them at 121°C for 20 min under 15 psi pressure.
3. Let the media cool upto 40°C , and then add 100 mg naringin in sterilized minimal agar media. Rotate flask clockwise and anticlockwise for uniform distribution of naringin (*see Note 2*).
4. Pour it into the sterile petri plates and keep it at room temperature for 2–3 h for solidification. Also prepare control plates of each culture media but without naringin.
5. Take loopful of soil suspension from 10^{-5} to 10^{-6} dilution tube and streak on naringinase agar (*see Note 3*).
6. Incubate the plates at room temperature up to approximately 48 h, observe the plates for growth with zone of dissolution.
7. Compare the test plates with the control plates to examine the exact naringinase production and formation of zone of dissolution.
8. **Confirmation of naringinase producers**

A loopful of microbial culture streaked on Modified Minimal Davis (MDN) medium supplemented with naringin as a sole carbon source (isolated bacteria, fungi, and actinomycetes) and incubated at $37 \pm 2^{\circ}\text{C}$, $25 \pm 2^{\circ}\text{C}$ for 48 h. After incubation, the plates (without lid) were inverted and exposed

to iodine crystal vapors for 15–20 min and observed for the zone of clearance surrounding the microbial colony.

9. Quantitative estimation can be performed as per the method of Habelt and Pittner [5] naringinase assay or by analyzing produced reducing sugar as per Millers 1959, DNSA method (*see Note 4*).

4 Notes

1. Instead of naringin, many natural glycosides, which include rutin, quercitrin, hesperidin, diosmin, and ter-phenyl glycosides, containing terminal α -L-rhamnose and β -D-glucose can be used as substrates of naringinase composing the naringinase isolation agar.
2. Avoid adding naringin into the media composition preparation before autoclaving, as this may result into degradation or loss of substrate due to the heat and pressure. Add Naingin solution prepared in methanol or 70% ethanol in sterile minimal medium prior to the addition of microbial culture.
3. For the rhizobium and the rhizospheric endophytes, surface sterilizes the material with 70% ethanol or 0.01 mercuric chloride washing. Then crush the sample in sterile saline, further dilute and streak specific dilution on sterile naringinase agar medium.
4. Determine naringinase unit by enzyme assay of Habelt and Pittner [5] assay method. Take 400 μ l of 0.1% (w/v) substrate naringin dissolved in 0.1 M sodium acetate buffer (pH 4.0) in clean glass test tubes, add 100 μ l of supernatant of potent culture, incubate for 1 h above 40 °C. Take this 100 μ l of the reaction mixture in test tube and add 3 ml 4 M aqueous NaOH. Mix well and incubate at room temperature for 20 min. The yellow color will be developed which is measured for absorbance at 310 nm, i.e., naringenin [6]. Also determine produced reducing sugar by DNSA methods of Millers 1959 [7]. One unit (U) of naringinase activity is equal to produce 1 μ mol of naringenin or glucose per minute.

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Isolation and Screening of Phytase Producing Microorganisms: An Essential Bioinput for Soil Fertility

Bhavana V. Mohite, Kiran Marathe, Narendra Salunkhe, and Satish V. Patil

Abstract

Phytase is one of the identified enzymes which plays a vital role in phosphorous (P) cycle by mobilization of immobilized P from plant material. Although there is various phosphate solubilizing biofertilizer agents reported, they are majorly targeted for mobilizing rock phosphates in soil. The Phosphate solubilizing biofertilizers are required for mobilizing the immobilized form of P from plant source i.e., from phytate. The protocol is based on the principle that specific phytate agar medium contains calcium/sodium phytate, which is utilized as P source by phytase producing microorganism indicated by zone of clearance around the colony. The further quantification is executed by measuring the free phosphate by spectrophotometric method.

Key words Phytase, Phosphate, Phosphorous, Phytate, Macronutrient

1 Introduction

Phosphorous (P) is one of the major macronutrients for plant growth. It contributes approximately 0.2% of plant's dry biomass. It is important component in nucleic acid, ATP, phospholipids, etc. which contribute for plant growth and metabolic activity. P determines many vital activities like development of flower, seed development, germination and maturity, and consequently majorly affects the crop maturity and yield [1]. Besides these, various vital activities like N-fixation in legumes, energy metabolism, synthesis of membranes, photosynthesis, respiration, enzyme regulation, crop quality, and resistance against various biotic and abiotic stress are also subject to P nutrition. Soil has always less utilizable form of P for plant; it warrants the application of P fertilizers in soil. The reaction with metals, absorption and precipitation of inorganic P in soil are major physical phenomenon which cause added fertilizers unavailable to plants. The high accumulation of unavailable phosphate also leads to eutrophication and infertility of soil. In plants, P

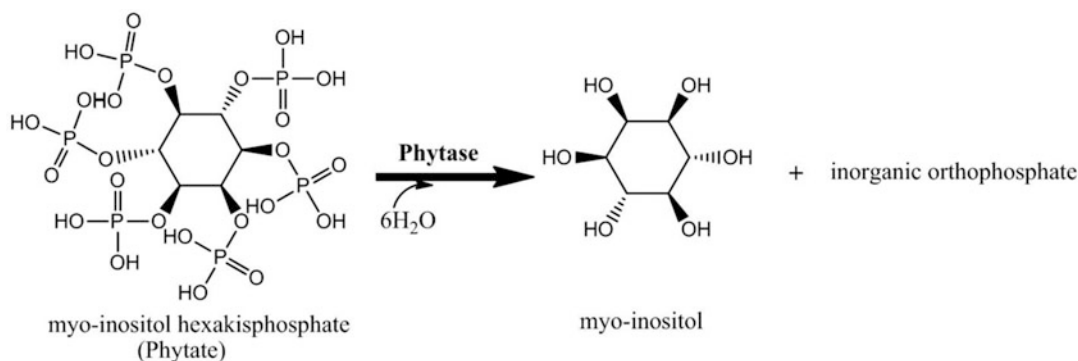


Fig. 1 Mechanism of phytase action for phytate solubilization. (Reproduced from Mohite et al. [3] with permission from Springer Nature)

is in organic form of phytate which forms salts with monovalent and divalent cations like Fe^{2+} , Mn^{2+} , K^+ , Mg^{2+} , and Ca^{2+} , making it unavailable for assimilation [2]. Phytases are group of enzymes which naturally make this P available for recycling in nature (Fig. 1). Phytases are classified according to pH activity, catalytic mechanism, source and initiation of site of dephosphorylation of substrate. As per IUPAC-IUBMB, phytase grouped in major three classes, i.e., 3-phytases (EC 3.1.3.8), 6-phytases (EC 3.1.3.26), and 5-phytases (EC 3.1.3.72), which further divided as acid and alkaline phytases on basis of optimum pH [4] (Fig. 2). As per recent reports, microbial phytases are considered as new bioinputs for soil as various fungi and bacteria utilize phytate as substrate and make available in assimilable form to crops or plants [5, 6].

1.1 Principle of the Assay

The microorganism having phytase producing capacity will utilize phytate as phosphorus source and indicate by calcium/sodium phosphate dissolution. The phytate dissolution by organic acid will be differentiated by flooding cobalt chloride, ammonium molybdate, and ammonium vanadate solution, these agents reprecipitate the acid solubilized phytases, and the zone due to organic acid dissolution will be disappeared (*see Note 1*).

2 Materials

All the culture media used are from HiMedia, Mumbai including the sodium selenite. Pure grade distilled water was used for dilution.

1. Calcium or sodium phytate.
2. 0.2 M sodium acetate buffer (pH 4.0).
3. Trichloroacetic acid.

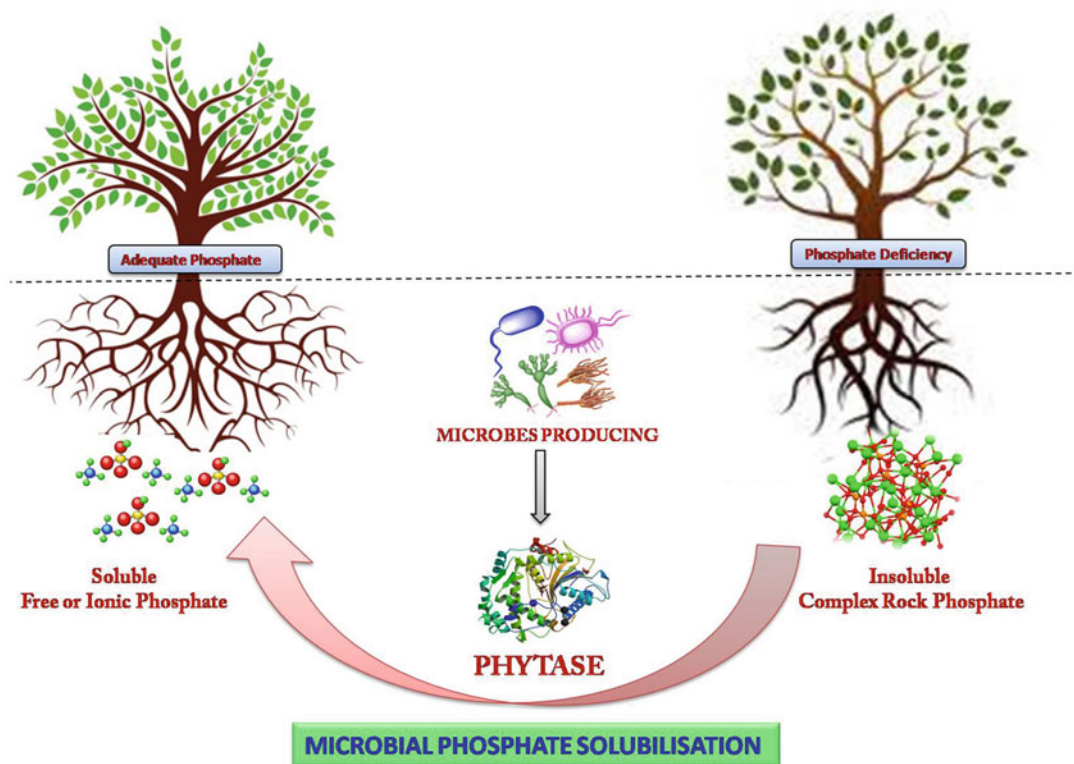


Fig. 2 Microbial phosphate solubilization. (Reproduced from Mohite et al. [3] with permission from Springer Nature)

4. 100 mM glycine/HCl buffer (pH 2.5).
5. Rhizospheric soil sample from the agricultural crops such as wheat or maize and root nodules of the bean crops.
6. **Phytase Isolation agar** (g/l): Glucose 10.0, Calcium Phytate 1.0, Ammonium nitrate 0.1, KCL 0.5, NH_4NO_3 0.5, $\text{MnSO}_4 \cdot 7\text{H}_2\text{O}$ 0.01, and $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ 0.01. Dissolve in slightly hot water, add 3.0 g agar agar powder, and sterilize at 21 °C for 15 min.
7. 10 mM sodium phytate and 100 mM glycine/HCl buffer (pH 2.5).
8. Confirmation reagents: **Reagent A**—2% (w/v) aqueous cobalt chloride solution and **Reagent B**—10 ml (25% (w/v) ammonium molybdate + 10 ml 0.42% (w/v) ammonium vanadate solution.
9. Ammonium molybdate solution (AMS): 1.5 g ammonium molybdate in 5.5% sulfuric acid.
10. 2.7% Ferrous sulfate w/v.

11. Reagent for phosphate estimation: 4 ml of AMS + 1 ml of ferrous sulfate solution.

3 Methods

1. Take 1.0 g of collected soil sample, add in 9.0 ml sterile saline, mix well by shaking and then further dilute up to ten folds, i.e., 10^{-9} .
2. Prepare phytase agar by using pure distilled water in clean 250 ml Erlenmeyer flasks and sterilize them at 121 °C for 20 min under 15 psi pressure.
3. Let the media cool at 40 °C, pour it into the sterile petri plates and incubate it at room temperature for 2–3 h, for solidifying. Also prepare control plates of each culture media but without calcium phytate.
4. Take loopful of soil suspension 10^{-5} to 10^{-6} dilution tube and streak on phytate agar plates.
5. Incubate the plates at room temperature up to approximately 48 h, and observe that the plates for growth appear with hollow zone around colonies.
6. Compare the phytate containing plates with the control plates, colonies showing high zone of dissolution, subculture it for further study.
7. The confirmation of phytase production, i.e., phytate solubilization due to phytase but not by organic acid or other metabolic product, could be confirmed by counterstaining treatment [7]. In brief, do spot inoculation of potent phytase producer on sterile phytate agar, and allow growing at 48 h as per organism optimum incubation period. After incubating flood plate with reagent A (2% cobalt chloride solution), incubate for 5 min. Remove extra solution from the plate and overlay the petri plates with Reagent B (ammonium molybdate solution and ammonium vanadate). Incubate plates for 5 min. Observe for the zone of clearance (*see Note 2*).
8. The zone of dissolution after counterstain indicates the real phytate dissolution by phytase.
9. **Quantitative assay:** In quantitative phytase assay, the active culture grow in 0.1% sodium phytate containing medium for 24 h, and centrifuge supernatant as 10,000 lb for 10 min [8]. The crude enzyme in supernatant is collected. Add 1 ml of supernatant broth in 600 µl of sodium phytate solution, incubate at 37 °C for 30 min. Terminate the reaction by adding 700 µl of 5% TCA solution. After that take 1 ml of this reaction mixture, add 0.5 ml AMS reagent. Thereafter phytase activity is

determined as micromole inorganic phosphate release per min at 700 nm by using standard graph of K_2HPO_4 (*see Note 3*).

4 Notes

1. The zone after counterstaining avoids false results of phytate dissolution and pseudo phytase activity. The colony shows dissolution on phytase agar but not after counter staining confirm that phytate utilization is not either by metabolic product of media utilization or by organic acid production.
2. Prepare counterstain **Reagent A**, i.e., (cobalt chloride solution) and **Reagent B** (ammonium molybdate solution and ammonium vanadate mixture) in distilled water.
3. One unit phytase activity (U/mL) was defined as the amount of enzyme required to liberate 1 μ mol of inorganic phosphate per minute.

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Part V

Identification of Compounds Involved in Biocontrol Activity



Hydrogen Cyanide

Khyati Bhatt and Dhruti Amin

Abstract

Hydrogen cyanide is a volatile compound synthesized by plants as well as microorganisms including fungi and bacteria. Biological control of plant pathogens is gaining momentum since a few decades due to the hazardous nature of chemical pesticides to living organisms and the environment. Various fungal and bacterial organisms act as antagonists and induce the disease resistance in the host organism by producing various compounds. One such important compound that imparts the antagonist properties to a wide range of plant growth promoting bacteria and other microorganisms is hydrogen cyanide. Many researches have attempted various ways for isolation of hydrogen cyanide producing organisms from the soil and their role in biocontrol activity in a variety of plants. The majority organisms producing HCN are plant growth promoting rhizobacteria and endophytes.

Key words Hydrogen cyanide, Biocontrol, PGPR, In vitro production

1 Introduction

Biocontrol of wide range of pathogens is an emerging field of study that provides us with the natural, effective, and environment friendly method of mitigating the diseases and inducing disease resistance in plants with improved growth and performance. The role of soil microbes, probably the bacteria present in the rhizosphere as plant growth promoting bacteria became evident during late 1800s presence of plant growth promoting bacteria in the soil is reported by researchers [1]. These microorganisms are known to govern the developmental processes, provide biocontrol from broad range of phytopathogens, and help in nitrogen fixation and availability of nutrients [2, 3]. These microorganisms have the ability to produce many antimicrobial compounds including the volatile metabolite hydrogen cyanide (HCN) [4]. Some of the plant growth promoting bacteria responsible for HCN production include *Pseudomonas*, *Azotobacter*, and *Rhizobium* [5–7]. These cyanogenic organisms produce hydrogen cyanide which disrupts the cell of other organisms by inhibiting essential components of

respiratory chain and synthesis of other important enzymes. HCN can also increase phosphate solubility and forms complex with iron which creates iron deficiency for competing microorganisms [5–8]. The process of cynogenesis in bacteria is initiated by the precursor glycine which undergoes oxidative decarboxylation to produce HCN and CO_2 by enzyme HCN synthase [9–11]. This chapter demonstrates common method of isolation of HCN producing bacteria, qualitative and quantitative characterization of HCN from bacteria, and in vitro method for analyzing inhibiting property of HCN producing bacteria on pathogenic microorganisms.

2 Materials

2.1 Isolation of PGPB from Soil [12]

1. Rhizosphere soil.
2. Zip lock sterile bag for collection of samples.
3. Spatula and wireloop.
4. Petri plates and sugar tubes.
5. Glycerol.
6. Distilled water.
7. King's B agar (KBA) medium (20 g L^{-1} peptone, 1.5 g L^{-1} K_2HPO_4 , $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 10 g L^{-1} agar).
8. Shaker.
9. Incubator.

2.2 Screening of HCN Producing Bacteria [13]

1. Nutrient agar (supplemented with sucrose/glucose).
2. Glycine ($4/4 \text{ g L}^{-1}$).
3. King's B broth medium.
4. Whatman filter paper no. 1 sterilized and soaked in picric acid solution (0.5% picric acid and 2% Na_2CO_3).
5. Parafilm.
6. Incubator (30°C).
7. Erlenmeyer flask/Conical flasks.

2.3 Quantitative Analysis of HCN Production

2.3.1 Sodium Picrate Method [14]

1. King's B broth medium.
2. Sterile Whatman filter paper no. 1 and soak in picric acid solution (0.5% picric acid and 2% Na_2CO_3).
3. Glycine ($4/4 \text{ g L}^{-1}$).
4. Parafilm.
5. Distilled water.
6. Erlenmeyer flask/conical flasks, sugar tubes, pipette.
7. Spectrophotometer.

2.3.2 Methemoglobin Method [15, 16]

1. Luria–Bertani broth.
2. Glycine (5 g L^{-1}).
3. Hemoglobin, NaNO_2 , NaCl , KCl , Na_2HPO_4 , KH_2PO_4 .
4. Parafilm.
5. Distilled water.
6. Erlenmeyer flask/conical flasks, sugar tubes, pipette.
7. Incubator, UV-Vis Spectrophotometer.

2.4 Inhibition of Phytopathogens by HCN Producing Microorganisms [17]

1. King's B agar and broth media/nutrient agar/potato dextrose agar media.
2. Petri plates, Erlenmeyer flask/conical flasks, sugar tubes, pipette, glass spreader, wire loop.
3. Whatman filter paper no. 1.
4. Laminar airflow.
5. Incubator (28°C).

3 Methods

3.1 Isolation of PGPB from Soil [12]

1. Collect the soil from rhizosphere, near roots of plants into airtight zip lock bag and transfer to lab. Store samples at 4°C until further use (*see Note 1*).
2. Mix 10 g of soil sample in an Erlenmeyer flask containing 95 mL of distilled water. Mix the components by shaking the solution at 150 rpm for 15 min.
3. Dilute the above solution until 10^{-8} dilution is obtained (serial dilution method).
4. Streak 100 μL of the dilutions on King's B agar media plates and incubate at 30°C for 3 days.
5. Observe the bacterial colonies under UV light.
6. Select the bacterial colony which emits fluorescence and sub-culture on fresh King's B agar media plates.
7. Repeat the process until pure culture of the bacteria is obtained.

3.2 Screening of HCN Producing Bacteria [13] (*see Note 4*)

1. Prepare the nutrient agar media plates by adding $4/4 \text{ g L}^{-1}$ glycine or 100 mL of King's B broth in a 250 mL flask and add $4/4 \text{ g L}^{-1}$ glycine to the media.
2. Prepare control without glycine (*see Note 2*).
3. Streak/inoculate the loop full of isolated pure culture of bacteria on the NA plate/Kings B broth.
4. Dip the sterile Whatman filter paper no. 1 in picric acid solution.

5. Stick the filter paper on the inside of lid of Petri plate using parafilm. Close the lid and seal with parafilm or in case of using broth medium attach the filter paper to the neck of the flask and seal the mouth of flask using cotton plug and parafilm.
6. Incubate the plates/flask at 30 °C for 4 days.
7. Change in color of filter paper from yellow to light brown as weak (+), brown moderate (++) or reddish brown as strong (+++) indicate HCN producing bacteria followed by quantitative analysis as shown in Subheadings 3.3.1 and 3.3.2 (see **Note 3**).

3.3 Quantitative Screening of HCN Production

3.3.1 Sodium Picrate Method [13, 14]

1. Follow the steps from 1 to 7 of Subheading 3.2 as shown above.
2. Elute the color by dipping the strip (filter paper which shows color change from yellow to reddish brown) into sugar tube containing 10 mL distilled water.
3. Read the absorbance at 625 nm.
4. Use distilled water as blank.

3.3.2 Methemoglobin Method [15, 16]

1. Inoculate the bacterial pure isolates in Luria–Bertani broth supplemented with 5 g L⁻¹ glycine (pH 7.4). Keep the formation overnight.
2. Prepare the methemoglobin reagent by dissolving 0.34% (w/v) hemoglobin in 4 mM NaNO₂.
3. Incubate the mixture for 10 min.
4. Prepare phosphate buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄).
5. Mix methemoglobin reagent and phosphate buffer in 1:1 ratio and incubate at room temperature for 30 min.
6. Take 1 mL of the overnight bacterial cultures and 1 mL of mixture solution, i.e., methemoglobin reagent and phosphate buffer and incubate the mixture for 30 min.
7. Measure the absorbance at 427 nm. The concentration of HCN produced is expressed in μM.
8. Use KCN solution as standard.

3.4 Inhibition of Phytopathogens by HCN Producing Microorganisms [17]

1. The culture of any phytopathogenic bacteria or fungi can be used for bioassay.
2. Inoculate 3 mL (10⁸ cfu/mL) pathogenic bacterial suspension on solid King's B agar plate followed by proper spreading of suspension.
3. For fungal phytopathogens, pour potato dextrose agar media in sterile Petri plates. After solidification, spread 2–3 mL of spore suspension of pathogenic fungi using glass spreader (Add the loop of spores/portion of sporulating mycelium of fungi into 5 mL distilled water with 1–2 drops of tween 20 or 80).

4. Place a loopful of HCN producing bacteria directly on the center of the plate or a small disk of sterile filter paper dipped in HCN producing bacterial suspension can be placed in the center of the disk. This step is common for both bacterial and fungal phytopathogens.
5. The plates are incubated at 28 °C for 3–4 days.
6. Record zone of inhibition surrounding the HCN producing bacteria.
7. The larger the inhibition zone, better the antimicrobial activity.

4 Notes

1. Rhizosphere soil from the plants whose roots possibly harbor plant growth promoting bacteria for example, legumes and monocots such as maize and sugarcane can be used. Several endophytes are also reported to produce varying concentrations of HCN.
2. In abovementioned method of in vitro inhibition of pathogens by HCN producing bacteria, the medium used for the experiment should be selected depending upon the requirement of both the biocontrol agent and the pathogenic organism.
3. Staining and biochemical methods for bacteria identification refer standard microbiology laboratory manual.
4. The methods may need modification based upon the microorganism used for study.

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Chapter 47

Siderophores

Nafisa Patel

Abstract

Siderophores are low molecular weight, metal chelating compounds produced by microorganisms in iron limiting stress conditions. These are classified based on functional groups and the types of microorganisms producing them. Media with different chemical composition are designed for the production of siderophores. CAS agar media is widely used to detect and screen siderophore producers, while the quantification is done by CAS shuttle assays and modified microplate assay method. The typing of the siderophore into hydroxamate, carboxylate, and catechols can be determined by tetrazolium test, iron perchlorate test, Csaky assay, iron perchlorate test, Arnow's test, and spectrophotometric test. Thin-layer chromatography and atomic absorption spectroscopy is applied for the characterization of purified siderophores.

Key words Siderophores, CAS, Iron-free media, Hydroxamate, Carboxylate, Catechols, AAS

1 Introduction

Microorganisms have high adaptability to withstand environmental changes due to their versatile metabolism. Many of these microbes when exposed to low iron stress conditions, develop strategies to bind iron by synthesizing low molecular compounds known as siderophores [1]. These are metal chelating compounds produced specifically under iron limiting conditions by bacteria, cyanobacteria, phytoplankton, and plants. These compounds take up iron and be complexed with other essential elements such as Mn, Mo, Ni, and Co, enabling their bioavailability to the microbial cells. Siderophore production increases with the increase in iron concentration, but an increase beyond a certain concentration tends to inhibit siderophore production [2]. Siderophores can be classified based on functional group and chemical nature of their coordination sites with iron, viz. hydroxamates, catecholates, and carboxylates. Some siderophores are phenolates, and others as mixed (both hydroxamate and catecholate functional groups). Siderophores can be typed based on the source, viz. bacterial, fungal, and cyanobacterial siderophores. The chrome azurol S (CAS) assay is a universal

colorimetric method that detects siderophores independent of their structure. In this assay, siderophores scavenge iron from a Fe-CAS-hexadecyltrimethylammonium bromide complex, and subsequent release of the CAS dye results in a color change from blue to orange. Siderophores are characterized in terms of their type, structure, and function existing in the different microbial population. More studies improve the understanding regarding its role in environmental applications such as bioremediation and biocontrol [3].

2 Materials

2.1 Media for Siderophore Production

1. CAS agar media.
2. MM9 media.
3. MEB media.
4. TYES media.
5. 8-hydroxyquinoline.

2.2 Inoculation of Media

1. 10^8 cells or spores/ml.
2. Rotary shaker.
3. Centrifuge.
4. Incubator.

2.3 Screening for Siderophore Producers

1. Chrome Azurol S (CAS) agar medium.
2. Bacterial isolates.

2.3.1 Bacterial Siderophores Producers

2.3.2 Fungal Siderophores Producers

1. CAS agar half plate.
2. Fungal isolates.

2.4 Quantitative Estimation of Siderophore

1. Culture.
2. CAS reagent.
3. Spectrophotometer.

2.4.1 Siderophore Estimation (CAS Shuttle Assay)

2.4.2 Modified Microplate Method

1. Culture.
2. CAS reagent.
3. Microplate.

2.5 Bioassays of Siderophores

1. Culture.
2. TYES media.
3. Iron starved indicator strains.
4. Petri plate.

2.6 Detection of Siderophore

2.6.1 Detection of Hydroxamate Siderophore

1. Culture.
2. 2% FeCl_3 .
3. 6 N H_2SO_4 .
4. 35% NaOAc .
5. I_2 solution.
6. α -naphthylamine.
7. Iodonitrotetrazolium.
8. NaOH .

2.6.2 Detection of Catechol Siderophores

1. Culture.
2. 2% FeCl_3 .
3. Nitrite molybdate.
4. 0.5 N HCl .
5. 1 N NaOH .

2.6.3 Detection of carboxylate Siderophores

1. Culture.
2. 250 M CuSO_4 .
3. Acetate buffer (pH 4.0).

2.7 Extraction, Purification and Characterization of Siderophore

2.7.1 Extraction of Siderophore

1. Culture.
2. Chloroform.
3. Phenol.
4. Ethyl acetate.
5. Acetic acid.
6. Formic acid.
7. Water.
8. Methanol.
9. Ca^{+2} (10 mM CaCl_2), Mg^{+2} (10 mM MgCl_2), PO_4^{+2} (10 mM K_2HPO_4), EDTA (100 mM Na_4EDTA), Hydroxylamine (0.5 M NH_2OH).
10. DMSO.

2.7.2 Purification of Siderophore

1. Column.
2. 0.1% NaNO_3 .
3. Methanol.
4. H_2O .

2.7.3 Characterization of *Siderophore*

1. Liquid chromatography electron spray ionization-mass spectrometry.
2. Acetonitrile and water (1:1 v/v) with 0.5% acetic acid.
3. TLC plates.
4. FeCl_3 .
5. Chloroform: methyl alcohol (90:10 w/v).
6. Atomic absorption spectrophotometer.

3 Methods

3.1 Media for *Siderophore* Production [4] (see Note 1)

1. MEB media: Malt Extract (2%), pH 5.6.
2. CAAB media g/l: Cas-amino acid (5.0), K_2HPO_4 (1.18), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.25), pH 5.6.
3. BRB media g/l: K_2HPO_4 (0.1), KH_2PO_4 (3.0), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.2), $(\text{NH}_4)_2\text{SO}_4$ (1.0), Succinic acid (4.0), pH 5.6.
4. SMB media g/l: K_2HPO_4 (6.0), KH_2PO_4 (3.0), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.2), $(\text{NH}_4)_2\text{SO}_4$ (1.0), succinic acid (4.0), pH 5.6.
5. G-A media g/l: K_2SO_4 (1.0), K_2HPO_4 (3.0), Ammonium acetate (3.0), Citric acid (1.0), sucrose (20.0), pH 6.8.

From the above list, any media can be used for siderophore production.

3.2 Inoculation of Media [5]

1. Inoculate 10^8 cells or spores/ml of 5–7 days old cultures into 150 ml TYES medium.
2. Incubate at their respective growth temperatures on a rotary shaker at 180 rpm.
3. Then centrifuge 10–12 days old cultures at 10,000 rpm for 15–20 min.
4. Examine supernatants for extracellular siderophore production.

3.3 Screening for *Siderophore* Producers [6]

3.3.1 Bacterial *Siderophores* Producers

1. Prepare Chrome Azurol S (CAS) agar medium.
2. Inoculate ME agar medium with Chromo Azurol S (CAS) (blue agar) with 24 h old bacteria.
3. Incubate at 30 °C for 72 h.
4. Observe a change of blue color of the medium to orange or presence of yellow to light orange halo surrounding the colony indicates the production of siderophore.

3.3.2 Fungal Siderophore Producers

1. Prepare universal CAS assay with modification (CAS agar half plate).
2. Prepare Petri dishes (10 cm in diameter) with the MEA medium and let it solidify.
3. Cut the medium into halves and replace one half by CAS blue agar.
4. Inoculate the inoculums at extreme ends of each other.
5. Incubate the plates in the dark at 28 °C for 6 days.

3.4 Quantitative Estimation of Siderophore [7, 8]

3.4.1 Siderophore Estimation (CAS Shuttle Assay)

1. Inoculate the media with the test culture and incubate at appropriate conditions.
2. Mix 0.5 ml of culture supernatant with 0.5 ml of CAS reagent.
3. Measure absorbance at 630 nm against a reference consisting of 0.5 ml of uninoculated broth and 0.5 ml of CAS reagent.
4. Calculate siderophore content using following formula as percent siderophore unit (psu):

$$\% \text{Siderophore units} = \frac{Ar - As \times 100}{Ar}$$

where

Ar = Absorbance of reference at 630 nm (CAS reagent)

As = Absorbance of sample at 630 nm.

3.4.2 Modified Microplate Method [9]

1. Inoculate the media with the test culture and incubate at appropriate conditions.
2. Add 100 µl of each test culture in separate wells of microplate.
3. Add 100 µl of CAS reagent and incubate.
4. Measure the optical density of each sample at 630 nm using microplate reader.
5. Calculate the siderophore content by the same formula as shown in Subheading 3.4.1.

3.5 Bioassays of Siderophores [10]

1. Procure specific indicator strains for the various types of siderophores. (*Arthrobacter flavescens* JG9 for hydroxamates, *Salmonella typhimurium* *enb7* for catecholates, and *Morganella morganii* SBK3 for carboxylates).
2. Grow the non-siderophore indicator strains in iron-free media.
3. Inoculate the iron starved indicator strains in a Petri plate containing TYES media.
4. Soak the filter paper disks with the supernatant of the test organisms and place it on the TYES media inoculated with the indicator organisms.
5. Incubate the plates at appropriate temperature for 24–48 h.

6. Observe for the presence of visible growth surrounding the disk. Growth indicates siderophore production by the test organism.

3.6 Detection of Siderophores [11–14]

3.6.1 Detection of Hydroxamate Siderophore

Iron Perchlorate Test

1. Collect 1 ml of filtrate from 15 days old production media.
2. Add 1–5 ml of freshly prepared aq. 2% FeCl_3 solution to the filtrate.
3. Measure the development of orange color with absorbance range of 200–60 nm.
4. Absorbance peak at 420–450 nm indicates presence of ferric hydroxamate siderophores.

Csaky Assay

1. Collect 1 ml filtrate from 15 days old production media.
2. Hydrolyze it with 1 ml 6 N H_2SO_4 in boiling waterbath for 6 h.
3. After hydrolysis, add 3 ml 35% NaOAc to buffer the solution.
4. Add 0.5 ml I_2 (1.3 g/100 ml glacial acetic acid) and incubate for 3 min.
5. Remove excess I_2 by addition of 1 ml Na_3AsO .
6. Add 1 ml of α -naphthylamine and observe for any color development.
7. Wine red color development indicates hydroxamate type.

Tetrazolium Test

1. Collect 1 ml of filtrate from 15 days old production media.
2. Add mixture of two drops of 2 N NaOH to a pinch of iodoni-trotetrazolium (INT).
3. Observe the formation of color.
4. Development of the color indicates the hydroxamate siderophores.

3.6.2 Detection of Catechol Siderophores

Iron Perchlorate Test

1. Collect 1 ml filtrate from 15 days old production media.
2. Add 1 ml of freshly prepared aq. 2% FeCl_3 solution to the filtrate.
3. Observe for the development of wine color.
4. Absorbance peak at 495 nm indicates presence of catechol siderophores.

Arnow's Test

1. Collect 1 ml filtrate from 15 days old production media in screw cap tubes.
2. Add 1 ml of nitrite-molybdate reagent.
3. Add 1 ml of 0.5 N HCl to give yellow color solution.
4. Add 1 ml of 1 N NaOH solution, and formation of red color indicates catechol siderophores.

3.6.3 Detection of Carboxylate Siderophores

1. Collect 1 ml filtrate from 15 days old production media in screw cap tubes.
2. Add 1 ml of 250 M CuSO_4 and 2 ml of acetate buffer (pH 4).
3. Absorbance peak between 190 and 280 nm indicates presence of carboxylate siderophores.

3.7 Extraction, Purification, and Characterization of Siderophore (see Note 2)

3.7.1 Extraction of Siderophore

1. Collect filtrate from 15 days old production media.
2. Add appropriate amount of solvent and mix well.
3. Separate the solvent and extract the siderophore.
4. Solvent systems such as chloroform: phenol, ethyl acetate: acetic acid, formic acid: water, methanol: water.
5. Anions, cations, reductants or their combinations such as Ca^{+2} (10 mM CaCl_2), Mg^{+2} (10 mM MgCl_2), PO_4^{+2} (10 mM K_2HPO_4), EDTA (100 mM Na_4EDTA), Hydroxylamine (0.5 M NH_2OH).
6. Combination of ions and reductants such as ascorbate (10 mM/2.5 mM) can be used.
7. Re-extraction can be done with ethyl acetate + acetic acid/isopropanol/DMSO.

3.7.2 Purification of Siderophore

1. Centrifuge the broth at 5000 g for 15 min at 4 °C.
2. Concentrate the CAS positive supernatant on a rotary vacuum evaporator at 100 rpm at 50 °C (pH 6.0 adjusted with 12 N HCl).
3. Prepare Amberlite XAD-400 resin column by dissolving resin in distilled water and soak it overnight at 8 °C.
4. Treat the resin with 0.1% NaNO_3 and load in sintered glass column.
5. Pre-wash the loaded column with water, methanol, and water.
6. Add the aqueous concentrated supernatant having siderophores slowly in the column at the rate of 5 ml/min. Load the column till its saturation (browning of column).
7. Wash the columns five to ten times with distilled water to remove the unbound components.
8. Equilibrate the column for extraction.

3.7.3 Characterization of Siderophores [15]

Thin-Layer Chromatography

1. Spot the extracted siderophore on silica gel TLC plate.
2. Elute the plate with chloroform and methyl alcohol (90:10 v/v).
3. Spray the dried plates with 0.1 M FeCl_3 in 0.1 N HCl and air dry.
4. Observe the formation of spots.

Atomic Absorption Spectra (AAS) Analysis

1. Prepare samples by suspending extracted siderophore in 2% FeCl₃ solution. 2% FeCl₃ solution in water as control.
2. Determine the amount of dissolved Fe⁺³ using atomic absorption spectrometer.
3. Calculate the percent Fe⁺³ in siderophore suspended sample using formula:

$$\text{Total Fe}^{+3}\% \text{ with siderophore treatment} = \frac{\% \text{Fe}^{+3} \text{ in siderophore treated sample by AAS}}{\% \text{Fe in control by AAS}} \times 100$$

4. Calculate the % Fe⁺³ scavenged by siderophores using formula:

$$\% \text{Fe}^{+3} \text{ scavenged} = 100 - \% \text{Fe}^{+3} \text{ in siderophore sample with reference to control.}$$

Liquid Chromatography Electron Spray Ionization-Mass Spectrometry

1. Dilute the siderophore with acetonitrile and water (1:1 v/v) with 0.5% acetic acid and filter it.
2. Mass was measured in the range from m/z 50–1200 with a flow rate of 0.3 ml/min.
3. The parameters such as electron spray ionization (ESI), ion trap analyzer, ion polarity positive set nebulizer 1.8 bars, focus active capillary 3500v, and dry heater 180 °C use for Liquid Chromatography Electron Spray Ionization-Mass Spectrometry.

4 Notes

1. Soak all glassware overnight in 6 mol/l HCl and rinse several times with water to remove the iron traces in the glassware before using it for the siderophore experiment.
2. Solvent system for extraction is selected based on maximum extraction of siderophore.

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Isolation and Screening of ACC Deaminase-Producing Microbes for Drought Stress Management in Crops

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Abstract

The compound aminocyclopropane-1-carboxylic acid (ACC) is the product of Yang cycle, which plays a vital role in drought response, salt stress, host–pathogen interactions, seed germination, flowering, and fruit ripening. While ACC is produced within the plant, some of it is exuded from the roots where it may alter the rhizosphere microbial composition. Within the plant, ACC may be converted to the phytohormone ethylene by the action of the plant enzyme ACC oxidase (ACO). When the rhizosphere or the plant endosphere contains microbes that produce ACC deaminase, some of the ACC is converted to alpha ketobutyric acid and ammonia. Thus, reducing ethylene production and decreasing the negative effects of ethylene including numerous drought-like symptoms, thereby reduces the deleterious effects of drought stress. ACC deaminase-producing microorganisms may readily be isolated by using minimal medium containing ACC as sole source of nitrogen and a pH indicator. The microbial cleavage of ACC changes the pH of the growth medium and causes an easily detectable color change around the colony. Subsequently, qualitative or quantitative assays for alpha ketoglutaric acid or ammonia may be used to assess the level of ACC deaminase activity.

Key words PGPR, ACC deaminase, Screening, pH dye, Phenol red

1 Introduction

The studies of Yang and his colleagues pioneered the research to unlock the mystery of freshness of fruit, flowers, defoliations, the ripening of fruits, etc. through the Yang cycle [1]. Ethylene has a vital role in host–pathogen interactions, seed germination, flowering, fruit ripening, and the response of plants to both biotic and abiotic stress [2].

Yang's studies proved that the synthesis of S-adenosylmethionine is an intermediate compound which further converted to aminocyclopropane-1-carboxylic acid (ACC) and then, by the action of the enzyme ACC oxidase to ethylene (Fig. 1).

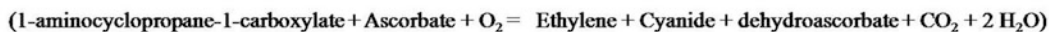
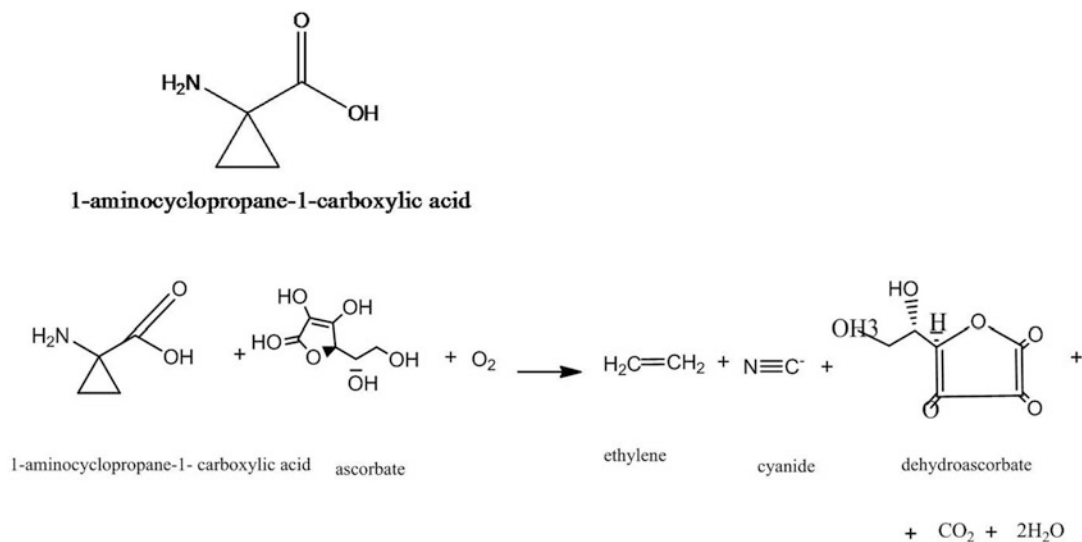


Fig. 1 Conversion of ACC into ethylene

In plants, ethylene level has been found to be rapidly increased following the onset of drought and other stress conditions [2, 3]. This ethylene synthesis is a marker of the stress condition and is sometimes known as stress ethylene.

1.1 Amino-cyclopropane-1-Carboxylic Acid Deaminase and Its Role

Glick et al. [4] described the role and importance of some plant growth promoting *Rhizobacteria* in management of drought stress and various physiological activities of plants. This work showed that the ACC is produced in greater quantities during drought stress; ACC is exudated outside by the root cells. The plant growth inducing bacteria around the roots are recognized for their various beneficial activities. These microbes utilize the plant exudates including ACC by using ACC deaminase which helps to maintain the balance between the inner and outer ACC level. As more of the ACC gets secreted outside the plant roots, it reduces the concentration of ACC that can be used for the synthesis of ethylene inside the plant cells. In this way by utilizing ACC, rhizospheric microbes help to reduce the ethylene production that occurs during drought conditions. This leads to less defoliation, more root elongation, increased nodulations, and increased transpiration activities [5] (Fig. 2).

Various rhizospheric microbes have been reported to have ACC deaminase-producing potential, e.g., *Enterobacter cloacae*, *Pseudomonas putida*, *Pseudomonas alcaligenes*, *Hansenula* spp., *Rhizobium*, *Sinorhizobium* spp., *Pseudomonas chlororaphis*, *Rhizobium leguminosarum*, *Bacillus subtilis*, *Penicillium citrinum*, etc. [6].

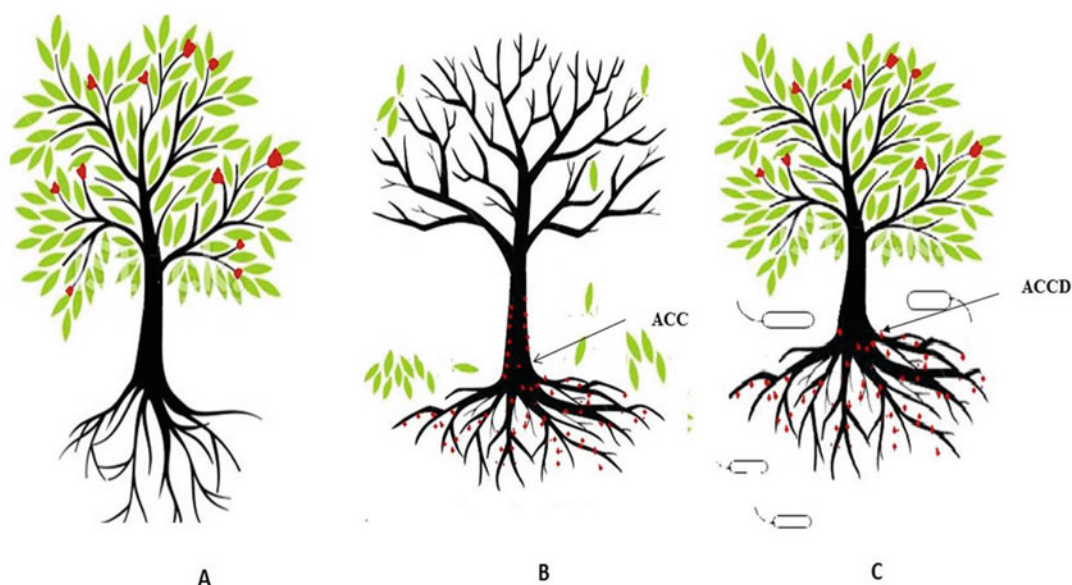


Fig. 2 ACC deaminase-producing microorganisms are attracted towards plants during stress conditions. (a) Normal plant, (b) Under the stress conditions the level of ACC increases and subsequent formation of ethylene causes defoliation and root growth inhibition. (c) The rhizospheric microorganisms utilize ACC in root exudates through the functioning of ACC deaminase and decrease the effects of the stress condition

ACC deaminase is a member of the oxidoreductase enzymes, a multimeric enzyme having monomeric subunit of molecular mass of 35–42 KDa [5]. ACC deaminase catalyzes the ACC to alpha ketobutyric acid and ammonia. It was reported that D-serine and D-cysteine can also act as substrates for ACC deaminase. ACC deaminase has a reported optimum pH and temperature of 8.5 and 30–35 °C, respectively [7–9].

1.2 Principle of Assay

This assay combines the two classical approaches as shown in Fig. 3, “Reproduced from Patil et al. [10] with permission from [Elsevier]” (1) Cultivation of test microorganisms on ACC-containing minimal medium [11] and (2) addition of pH indicator dyes phenol red and bromothymol blue.

The ACC deaminase-producing microbes utilize the synthetic substrate, ACC as a sole nitrogen source in minimal medium. The growing bacteria show a zone of clearance relative to its capacity for substrate utilization. The pH indicator dye shows a zone of color change around the colony due to the production of ammonia, a byproduct of the catalytic reaction of ACC deaminase.

Screening of ACC deaminase producers

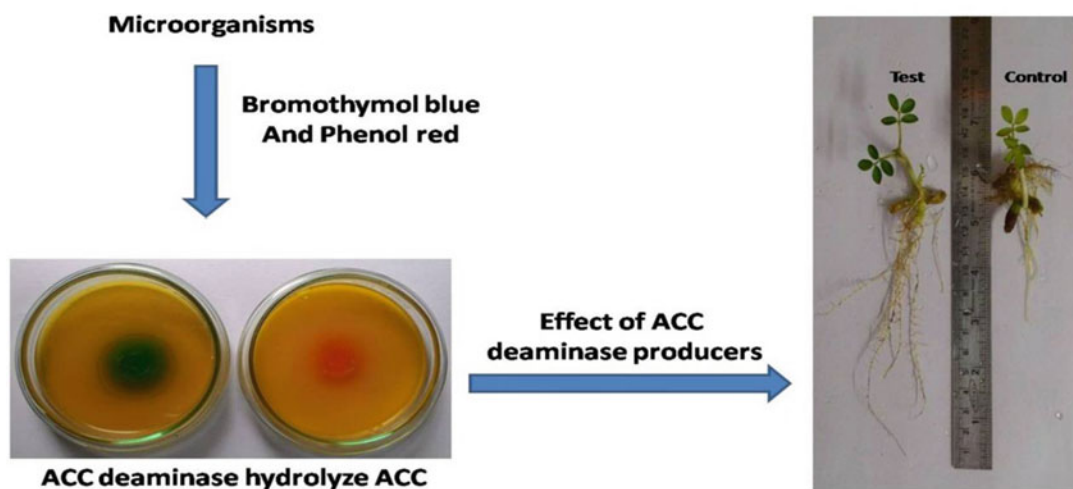


Fig. 3 Example of isolation and application of an ACCD producing microorganism and plant growth assay. ("Reproduced from Patil et al. [10] with permission from [Elsevier]")

2 Materials

2.1 Isolation of Rhizobacteria and Qualitative Estimation of ACC Deaminase Activity

1. Rhizospheric soil of plants, especially from drought areas.
2. Sterile plastic screw cap bottle.
3. Sterile saline tubes for serial dilution of the soil samples.
4. Sterile modified minimal medium.
5. Sterile Nutrient or LB agar.
6. Modified Dworkin and Foster salt medium.
7. Stock solution of trace elements.

2.2 ACC Deaminase Enzyme Assay

1. α -ketobutyrate.
2. 0.1% 2, 4-dinitrophenyl hydrazine solution.
3. 2 N sodium hydroxide.
4. 0.1 M Tris (hydroxymethyl amino methane) buffer (pH 7.5).
5. 0.56 N hydrochloric acid (HCl).
6. Trichloroacetic acid.
7. Nessler's Reagent.
8. Ammonium sulfate.
9. Potassium bromide.

3 Methods

3.1 Stepwise Procedure for the Isolation and Screening of ACC Deaminase-Producing Microbes from Soil

1. Prepare 100 ml of medium by mixing sterile modified minimal medium and the sterilized agar-agar solution, mix well by rotating flask and pour solution in plates.
2. Allow the plates to solidify.
3. Make serial dilutions of the test soil sample.
4. Streak the diluted soil suspension of dilutions 10^4 – 10^7 on solidified ACC plates, and incubate for 48 h at 37 °C.
5. Observe the color change around the colonies (Fig. 3) [10].
6. Subculture the pure colonies on nutrient or LB agar.
7. Make spot of diluted colony at the center of ACC plates and then incubate the plates for 48 h.
8. Measure the zone of color change as a function of time.
9. Compare the isolated colonies with the zone of color changes and note the colony characters.

3.2 Collection of Soil

For the isolation of ACC deaminase producers, rhizosphere soil of plants, especially from drought areas, should be collected in sterile plastic bottles. In addition, other rhizosphere soils from different locations may also be useful. Collect 1 g of rhizosphere soil in a sterile plastic screw cap bottle, close the bottle, label it, and store at 4 °C or freeze until further processing.

3.3 Isolation of Rhizobacteria and Qualitative Estimation of ACC Deaminase Activity

Dilute the collected soil sample up to 10^{-9} dilution in sterile 10 mL tubes, mix well and incubate for 10 min at room temperature. Then prepare modified Dworkin and Foster salt medium containing in g/l: Glucose, 2.0; KH_2PO_4 , 4.0; Citric acid, 2.0; Gluconic acid, 2.0; $\text{MgSO}_4 \cdot \text{H}_2\text{O}$ 0.2; Na_2HPO_4 , 6.0; and 3 mM amino cyclo propane, 594 ml; Distilled water to 1 l; mix this solution well.

Prepare stock solution of trace elements as follows: add $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 10 mg in 10 ml distilled water; 100 mg H_3BO_3 in 10 ml DW; 110 mg of $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ in 10 ml DW; 1240 mg $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ in 10 ml DW; 780 mg $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ in 10 ml DW; and 100 mg MoO_3 in 10 ml DW. Add 1 ml of each trace solution into above prepared Modified DF medium; maintain final pH of the medium 6.8–7.2. Then add 1 ml of bromophenol blue or phenol red into the medium mix, plug with cotton, and wrap with paper. To a separate flask, add 30 g of agar powder in 400 ml DW, mix well, plug the flask with cotton, wrap with paper and sterilize both preparations. After sterilization, allow the medium to cool to 40 °C and mix in agar solution into the modified DF medium and prepare plates.

3.4 ACC Deaminase Enzyme Assay

Preparation of standard curve of α -ketobutyrate: For quantitative assay, prepare stock of alpha-ketobutyrate (0.1–1.0 μ M) and add 0.3 ml of 0.1% 2, 4-dinitrophenyl hydrazine solution to it and incubate mixture at 30 °C, for 30 min. Thereafter add 2 ml of 2 N sodium hydroxide to the above mixture and measure the absorbance at 540 nm, and prepare a standard graph. For test organism capacity, estimate protein content of crude enzyme/fermented broth of isolate by the Bradford method and express activity by measuring the nano mole/1 μ M of alpha keto butyrate liberated per mg of cellular protein per hour.

3.4.1 ACC Deaminase Assay

For estimation of α -ketobutyrate, mix the enzyme extract (0.2 ml) and ACC (50 mM) and incubate in 0.2 ml of 0.1 M Tris (hydroxymethyl amino methane) buffer (pH 7.5) at 30 °C for 30 min. Terminate the reaction by adding 1.8 ml of 0.56 N hydrochloric acid (HCl). Add 0.3 ml of 0.1% 2,4-dinitrophenyl hydrazine solution and keep the mixture at 30 °C for 30 min. Thereafter, add 2 ml of 2 N sodium hydroxide in the above mixture and measure the absorbance at 540 nm. One unit of enzyme activity is defined as the “amount of enzyme liberating 1 μ M of α -ketobutyrate/min.” Perform the experiment at least in triplicate. (Reaction mixture can also be used for confirmation of activity by FTIR (*see* **Notes 1** and **2**).

3.5 Confirmatory Test for ACC Deaminase Production

In addition to α -ketobutyrate measurement, ACC deaminase production can be confirmed qualitatively and quantitatively by analyzing the release of ammonia from ACC. The procedure includes: Take a 100 μ l mixture of positive bacteria and 400 μ l 0.3 M ACC in Tris buffer solution and incubate at 37 °C for exactly 45 min. After the incubation add 250 μ l trichloroacetic acid 24.5% (w/w) and centrifuge the mixture at 50,000 rpm. Collect the supernatant and add 250 μ l of the supernatant to the Nessler's solution (2000 μ l water plus 250 μ l Nessler's reagent). Measure the optical density at 450 nm and compare the absorbance with an ammonium sulfate-Nessler's-standard curve based on daily calibration at concentrations of 0.05, 0.1, 0.25, 0.5, 1.0, and 2.5 mM. The units of enzyme activity are defined as micromoles of ammonia released per minute.

3.6 Quantitative Estimation

By using standard graph of α -ketobutyrate, extrapolate the reading of test sample (ACC deaminase-producing bacteria) and determine amount of α -ketobutyrate released where one unit of enzyme activity measured is the amount of enzyme liberating 1 μ g of α -ketobutyrate/min.

4 Notes

1. For confirmation of the α -ketobutyrate liberation after a deamination reaction by microbial ACC deaminase activity, the reaction mixture or broth may be used for potassium bromide

(KBr) cell pellet preparation and analysis with the help of Fourier-transform infrared spectroscopy (FTIR).

2. In FTIR spectra analysis results, observe peaks at 1689 and 3343 cm^{-1} , which confirm the presence of a ketonic group and amino functional group, respectively recognized as α -ketobutyrate [12].

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Exopolysaccharides

Sapna Chandwani and Natarajan Amaresan

Abstract

Certain microorganisms synthesize and excrete some essential metabolites known as exopolysaccharides (EPS) during adverse environmental conditions such as extreme pH, salinity, temperature, osmotic stress, salinity, or other contaminants. EPS protects the cell, helps the bacteria to adhere to solid surfaces and plays a role in cell-to-cell interaction. EPS determine the physicochemical properties of biofilm formation that help the bacteria attach to the substrate and develop biofilm formation.

Key words Exopolysaccharides (EPS), Biofilm formation, Mucoid colonies

1 Introduction

Multifunctional polysaccharides are synthesized by microbes which are high-molecular-weight polymers comprising sugar residues, including structural polysaccharides, intracellular and extracellular polysaccharides [1]. Microorganisms secrete and synthesize exopolysaccharides in two forms such as capsular EPS and slime EPS [2]. Generally, at the end of the growth phase, EPS production was maximum. During both the conditions—whether natural condition or laboratory condition, EPS production was maximum under nitrogen limiting conditions. Monosaccharides and some of the non-carbohydrate substituents like pyruvate, succinate, protein, lipids, nucleic acids, phosphate, etc. comprise together and form exopolysaccharides. A bacterial cell having a layer of EPS protects the cell from the adverse environmental conditions such as extreme pH, salt stress, osmotic stress, and extreme temperature stress [3].

EPS inhibit platelet aggregation, synthesize colony-stimulating factor and act as an inducer for interferon. EPS have antimicrobial, anti-inflammatory, and anti-tumor properties and help in biofilm formation and bioremediation by making a symbiotic relationship between plant and a microbe. These are biodegradable and

non-toxic and have various applications. For the industrial purpose, it has been used as thickeners, emulsifiers, stabilizers, gelling agents and in food industry used as a water binding agent [4, 5]. In the pharmaceutical industry, EPS is used as an active ingredient, as a microsphere vector for drug delivery, absorbent dressings as an active ingredient, and make dental impressions and anti-reflux therapies [6]. Multiple bacterial species secrete exopolysaccharides, thereby making biofilms a good source for screening EPS-producing bacteria [7, 8].

2 Materials

2.1 Collection of Sample

1. Soil sample/water sample or any of the sample.
2. Sterile plastic bag/sterile container.
3. Hand trowel.

2.2 Isolation and Purification of EPS-Producing Bacteria

1. Soil sample/water sample.
2. Sterile normal saline solution.
3. Vortex mixer.
4. Bushnell Hass agar medium.
5. Nutrient agar.
6. Nutrient broth.
7. Luria Bertini (LB) broth.
8. 2% sucrose.
9. Inoculation loop.

2.3 Isolation and Extraction of EPS

1. Culture inoculum.
2. Nutrient broth.
3. 2% sucrose.
4. Rotary shaker.
5. Refrigerator.
6. Centrifuge.
7. Pre-chilled ethanol or isopropanol.

2.4 Estimation of EPS by Phenol-Sulfuric Acid Test

1. Glucose.
2. Crude EPS obtained from sample.
3. 95% sulfuric acid (v/v).
4. 6% phenol.
5. Distilled water.
6. UV-visible spectrophotometer.

2.5 EPS and Biofilm Formation

1. Nutrient broth.
2. 1% glucose.
3. Phosphate buffer saline (PBS).
4. 1% crystal violet.
5. Distilled water.
6. Test tubes.
7. Rotary shaker.

3 Methodology

3.1 Collection of Sample

1. Collect the soil sample from 10 m depth below the soil surface with hand trowel into a sterile plastic bag and if it is a water sample then collect just below the water layer at the middle of the sea in a sterile container.
2. Transport the samples immediately to the laboratory, store it at 4 °C for further use.

3.2 Isolation and Purification of EPS-Producing Bacteria

1. Perform serial dilution method for isolating the bacteria from the samples.
2. Take 1 g of the soil sample or 1 ml of the water sample and suspend aseptically in 10 ml sterile physiological saline, homogenize it and label it as a stock solution.
3. Take 1 ml aliquot of stock solution, transfer it to 9 ml of sterile saline solution and vortex it.
4. Dilute the sample serially to 10^{-6} or 10^{-7} and then perform the spreading by inoculating 0.1 ml of each diluent suspension on Bushnell Hass agar medium or nutrient agar supplemented with 2% sucrose as carbon source and incubate it for 48 h for enumerating the bacteria.
5. Select the isolates forming thick mucoidal colonies and purify it on freshly prepared agar medium by streak plate method to obtain the distinct colonies.

3.3 Isolation and Extraction of EPS

1. Screen the isolates producing EPS maximum gravimetrically through the dry weight of exopolysaccharide produced by the isolate and quantitatively by phenol-sulfuric acid method.
2. Prepare a cell suspension of 1.5×10^8 bacteria/ml of each inoculum overnight grown culture and inoculate it in 50 ml nutrient medium supplemented with 2% sucrose as carbon source and incubate it on a rotary shaker at 30 °C for 3 days at 120 rpm.

3. Extract the exopolysaccharides from cell-free supernatant of the culture media with two volumes of the pre-chilled ethanol or isopropanol and precipitate it by refrigerating at 4 °C overnight.
4. Centrifuge at 15,000 rpm for 20 min and collect the precipitate and dry it at 60 °C.
5. Estimate the EPS in the form of pellet by measuring the total dry weight of the pellet obtained and perform the assay for total carbohydrate content by **phenol-sulfuric acid test**.
6. Select the bacterial strain producing the most significant yield for further study based on EPS dry weight and carbohydrate content values.

3.4 Estimation of EPS by Phenol-Sulfuric Acid Test [9]

3.4.1 Glucose Standard Curve Preparation for Quantitative Determination

2. Dissolve 4 mg glucose in water to make 100 ml.
3. Dilute the glucose solution with distilled water to 2 ml so as to make 4, 8, 12, and 16 mg/l glucose solution, respectively.
4. Add 1 ml of 6% phenol and 5 ml of 95% sulfuric acid (v/v) quickly and mix gently after 10 min standing.
5. Read the absorbance at 490 nm by UV-visible spectrophotometer using 2 ml of distilled water as blank.

3.4.2 Estimation of EPS

6. For EPS yield determination, weigh 1 ml of EPS sample and dilute to 2 ml with distilled water, then add 1 ml of 6% phenol and 5 ml of 95% sulfuric acid (v/v).
7. Shake well after 10 min standing and measure the absorbance at 490 nm.
8. Determine the EPS concentration in triplicate and use 2 ml distilled water as blank.
9. Calculate the EPS content of the sample by the standard curve.

3.5 EPS and Biofilm formation

1. Prepare nutrient broth medium having 1% glucose and inoculate it with isolated colonies.
2. Incubate the inoculated tubes for 24 h at 37 °C on the shaker to observe the organism's growth.
3. Decant the tubes and wash them with phosphate buffer saline to remove the bacteria and allow it to dry.

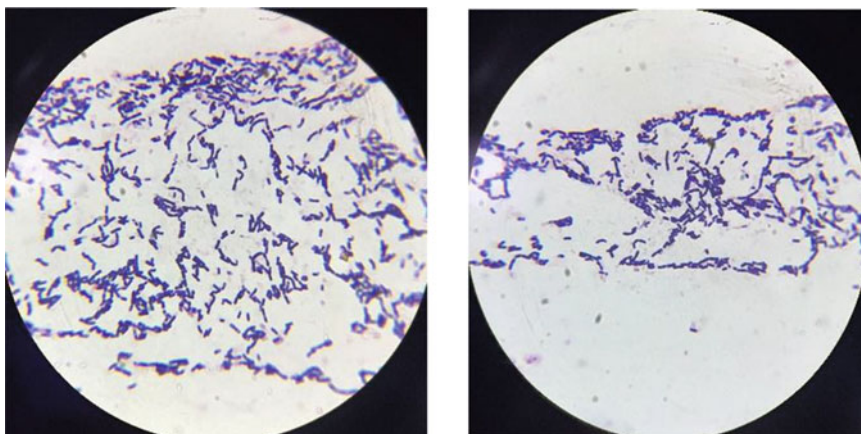


Fig. 1 Biofilm production by EPS-producing bacteria

4. Stain the dried tubes with 0.1% crystal violet solution and remove the excess stain using distilled water.
5. Dry the tubes in an inverted position and observe the biofilm production tubes (Fig. 1).

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Part VI

Assessment of Soil Microbial Activity by Soil Respiration



Enzymatic Analyses in Soils

Serdar Bilen and Veysel Turan

Abstract

Soil enzymes are included in the process of decomposition of the organic matter. The essential virtue of using enzyme assays for soil is that they are comparatively simple and mostly available analytical methods. However, since enzyme analysis results vary with the biological chemical, physical properties of soils, analytical methods may not reflect the true results. With the developing technology, the enzyme analysis methods used in soil enzyme analysis should be updated with new methods. The purpose of this section is to procure information regarding current methods that can be determined with new technologies in soil enzyme analysis.

Key words Acid and alkaline phosphatase activity, Amidase activity, Arylsulfatase activity, β -Glucosidase activity, Catalase activity, Dehydrogenase activity, Invertase activity, Phenol oxidase (Tyrosinase) activity, Phosphodiesterase activity, Urease activity

1 Introduction

The nutrient cycle in the soil includes biochemical and physico-chemical processes involving microorganisms, plant roots and soil functions [1, 2]. The main source of soil enzymes is microbial biomass, even they can also come from animal and plant residue [3]. Enzyme activities in the soil consist of free enzymes such as endoenzymes emerging from disintegrating cells, exoenzymes released from living cells and enzymes bound to cell components [4]. The intensity of the substrate in soil enzyme analysis should be specified at the peak value of enzyme activity, at the temperature and at the optimal pH [3]. Furthermore, in recent years, it is considered as an important indicator in determining the effects of heavy metals on soil health [5–8]. Consequently, soil enzyme activities have been attributed on gauging the rates of enzyme reactions and other biologically active compounds [9]. This section covers the up-to-date determination of analysis methods of some important enzymes researched in the soil.

2 Materials

2.1 *Materials for Acid (AcdP) and Alkaline (AlkP) Phosphatase Activity*

1. Spectrophotometer, Incubator adjustable to 37 °C, Filter paper (Whatman no. 2), Erlenmeyer flasks, Volumetric flasks (100 and 1000 ml).

2.2 *Materials for Amidase Activity*

1. Steam distillation apparatus, Temperature-controlled water bath, Volumetric flasks 50 ml, Incubator.

2.3 *Materials for Arylsulfatase Enzyme Activity*

1. Incubator, Filter paper (Whatman 2v), Stoppers, Funnels, Spectrophotometer or colorimeter, Erlenmeyer flasks (50 ml).

2.4 *Materials for β -Glucosidase*

1. Incubator adjustable to 37 °C, Water shaking bath, Erlenmeyer flasks (50, 100 ml), Vortex, Filter paper (Whatman 2v), Spectrophotometer, Glass funnel, Volumetric flasks (100, 500, 1000, 2000 ml).

2.5 *Materials for Catalase Activity*

1. Incubator adjustable volumetric flasks, (50 ml, 100 ml, 1 l), Glass tubes (15 ml), Erlenmeyer flasks (50 ml) or glass bottles (30–50-ml), Rotary shaker, Spectrophotometer, Shaking incubator, Vortex.

2.6 *Materials for Dehydrogenase Activity*

1. Incubator adjustable to 30 °C, Shaker, Vortex, Filter paper, Glass stick, Spectrophotometer, Glass funnel, Test tubes (60 ml volume, 2.5 cm diameter), Rubber stoppers, Volumetric flasks (50, 100, 1000 ml), Erlenmeyer flasks (50, 100 ml).

2.7 *Materials for Invertase Enzyme Activity*

1. Incubator adjustable to 30 °C, Volumetric flasks, Erlenmeyer flasks (50 ml) or glass bottles (30–50-ml), Vortex mixer, Rotary shaker, Shaking incubator, Spectrophotometer.

2.8 *Materials for Phenol Oxidase (Tyrosinase)*

1. Volumetric flasks (50 ml, 100 ml, 1000 ml), Shaker, Incubator, Shaking incubator, Nalgene centrifuge tubes (10 ml), Refrigerated centrifuge (12,000 rpm), Spectrophotometer.

2.9 *Materials for Phosphodiesterase Enzyme Activity*

1. Volumetric flasks (50 ml, 100 ml, 1000 ml), Rotary shaker, Erlenmeyer flasks (50 ml) or glass bottles (30–50-ml), Glass tubes (15 ml), Vortex, mixer, Spectrophotometer, shaking incubator.

2.10 *Materials for Urease Enzymes Activities*

1. Volumetric flasks (50 ml, 100 ml, 1000 ml), Steam distillation apparatus, Incubator, pH meter, Volumetric flasks (50, 100, 1000 ml), Automated titration, Erlenmeyer flask (50 ml, 100 ml).

3 Methods

3.1 Methods for Acid (AcdP) and Alkaline (AlkP) Phosphatase Activity [4, 10–14]

1. Add 1 g of each soil sample (<2 mm) in an Erlenmeyer flask (50 ml).
2. Transfer 0.2 ml of Toluene, 4 ml of Modified Universal Buffer MUB (0.17 M, pH 6.5 for AcdP, pH 11.0 for AlkP).
3. Transfer 1 ml of 0.025 M *p*-nitrophenyl phosphate (pNPP) solution (pH 6.5 for AcdP, pH 11.0 for AlkP) and rotate a few seconds.
4. Bung the flask, and transfer in the incubator for 1 h at 37 °C.
5. Transfer 1 ml CaCl₂ (0.5 M) and 4 ml NaOH (0.4 M).
6. Rotate 10–15 s for stopping the reaction, and filter it through with filter paper.
7. Read the yellow color concentration with a spectrophotometer at 405 nm.
8. To calculate the *p*-nitrophenol as µg pNP per gram dry matter. To check controls, follow assay of phosphatase activity, but make the addition of 1 ml of PNP solution after the additions of 0.5 M CaCl₂ and 4 ml of 0.5 M NaOH.

3.2 Methods for Amidase Activity [11, 15–17]

1. Put 5 g soil in 50 ml volumetric flask, and 9 ml of THAM buffer, add 0.2 ml of toluene.
2. Rotate solution for a few seconds, and mix.
3. Add 1 ml (0.5 M formamide solution), and rotate it for a few seconds.
4. Stopper the flask, and incubate the flask at 37 °C 2 h.
5. After incubation, add 35 ml of KCl-Ag₂SO₄ mix solution, rotate for a few seconds, and keep this solution at room temperature (apprx. 5 min).
6. Adjust the volume to 50 ml with KCl-Ag₂SO₄ mix solution, bung the flask, and mix.
7. Transfer a 20 ml piece of this mix solution into a 100 ml distillation flask.
8. Read the NH₄⁺-N released by distillation with 0.2 g of MgO for 4 min and calculate with titration. To check controls, follow assay of amidase activity, but make the addition of 1 ml of 0.5 M formamide solution after the additions of 35 ml of KCl-Ag₂SO₄ solution.

3.3 Methods for Arylsulfatase Enzyme Activity [4, 11, 18]

1. Add 1 g of field-moist soil (<2 mm) into 50-ml flask.
2. Add toluene (0.25 ml), acetate buffer (4 ml), and 1 ml of *p*-nitrophenyl sulfate solution (0.05 M).
3. Rotate for a few seconds to mix the content.

4. Stopper the flask and store it in the incubator at 37 °C.
5. After 60 min, remove the stopper, transfer 1 ml of 5 M CaCl₂ and 4 ml of 0.5 M NaOH.
6. Rotate for a few seconds and filter it.
7. Read the yellow color concentration with spectrophotometer at 420 nm.
8. To calculate *p*-Nitrophenol concentration, make a calibration curve. To check controls, follow for assay of arylsulfatase activity, 1 ml of 0.05 M *p*-nitrophenyl sulfate solution, after the additions of 1 ml of 0.5 M CaCl₂ and 4 ml of 0.5 M NaOH.

3.4 Methods for β -Glucosidase Enzyme Activity [4, 11, 19, 20]

1. Add 1 g of field-moist sieved (2 mm) soil into 50 ml flask.
2. Transfer toluene (0.25 ml), MUB solution (4 ml), 1 ml *p*-nitrophenyl glucoside (PNG), and then stopper the flasks.
3. Rotate the contents thoroughly and incubate for 1 h at 37 °C.
4. After the incubation, add 1 ml of CaCl₂ solution, 4 ml of Tris buffer, pH 12, rotate the flasks, and filter immediately.
5. Read the optical density of the clear supernatant at 400 nm with a spectrophotometer.
6. To check *p*-Nitrophenol ($\mu\text{g pNP g}^{-1} \text{ soil h}^{-1}$), prepare a calibration curve.
7. To calculate calibration curve, prepare 0, 10, 20, 30, 40, and 50 $\mu\text{g ml}^{-1}$ *p*-nitrophenol. After the incubation, follow the procedure described for assay of β -Glucosidase activity.
8. To check controls, follow the procedure described for assay of β -Glucosidase activity, but make the addition of 1 ml of CaCl₂ solution, 4 ml of Tris buffer, pH 12, after the additions of 0.25 ml of toluene, 4 ml of MUB solution, before incubation add 1 ml PNG solution.

3.5 Methods for Catalase Enzyme Activity [11, 21]

1. Put 0.2 g of soil (<2 mm) into four 50 ml glass vessels (3 for soil replicates and 1 for control).
2. Transfer 16 ml of distilled water to each vessel, and shake for 30 min on a rotary shaker at 30 rev min⁻¹.
3. Transfer 2 ml of 0.3% H₂O₂ solution, and shake it 10 min at 20 °C.
4. Transfer 2 ml 3 M H₂SO₄ to stop the reaction and filter it.
5. Transfer 1 ml of each standard (0–0.3% H₂O₂) solution and 1 ml 3 M H₂SO₄ to 8 ml distilled water.
6. After the filtration, pipette 100 μl aliquots and standards of the filtrates into 15 ml glass tubes and neutralize by adding 900 μl of 110 mM phosphate buffer.
7. Transfer 9 ml of indicator reagent to each tube and rotate by the vortex.

8. Wait for 5 min and read solution at 505 nm.
9. Calculate the content of H_2O_2 consumed as mmol g^{-1} dry soil h^{-1} .
10. To check control, for the assay of catalase activity, make the addition of 2 ml distilled water instead of 2 ml of 0.3% H_2O_2 solution. To check standards, transfer 1 ml of each standard (0–0.3% H_2O_2) solution and 1 ml 3 M H_2SO_4 to 8 ml distilled water and filter.
11. Take 100 μl aliquots of the assay filtrates and of the standards filtrates into 15-ml glass tubes and deactivate by the addition of 900 μl of 110 mM phosphate buffer. Transfer 9 ml of indicator reagent to each tube and rotate by the vortex.
12. The H_2O_2 solution in the standards and the enzyme assays was diluted tenfold before removal of the 100 μl aliquot and then the final H_2O_2 concentrations in the standards and assays range from 0 to 10 mM.

**3.6 Methods
for Dehydrogenase
Enzyme Activity [4, 11]**

1. Add 5 g of moist soil into test tubes.
2. Transfer 5 ml of TTC solution and rotate.
3. Seal the tubes with a rubber stopper and incubated for 24 h at 30 °C.
4. After the incubation, add 40 ml acetone to each tube, and shake thoroughly and incubate at room temperature for 2 h in the dark.
5. Filter the soil suspension (15 ml) and read the concentration of the clear supernatant against the blank at 546 nm.
6. Read the TPF concentrations ($\mu\text{g}/\text{ml}$) and calculate TPF concentrations as $\mu\text{g TPF dwt (g) soil}^{-1}$.
7. To check control, for the assay of dehydrogenase activity, make the addition of 5 ml Tris buffer solution instead of 5 ml of TTC solution.
8. To check calibration curve, make concentration 0, 5, 10, 20, 30, and 40 $\mu\text{g TPF ml}^{-1}$ with acetone.

**3.7 Methods
for Invertase Enzyme
Activity [3, 22, 23]**

1. Add 3 g of soil (< 2 mm) in a 50-ml Erlenmeyer flask.
2. Transfer 0.2 ml of toluene and 5 ml of acetate buffer and rotate for a few seconds.
3. Transfer 5 ml of the 10% sucrose solution (final concentration = 145 mM) and rotate the flask again for a few seconds.
4. Then stopper the flask and place it in an incubator at 37 °C for 24 h.
5. After incubation, remove the stopper and filter.
6. To determine the reducing sugars, pipette a 1-ml aliquot into a 50 ml test tube.

7. Transfer 5 ml of distilled water, 2 ml of 2 M NaOH, and 2 ml of the color reagent.
8. Pass a stream of N gas through the solution mixture for 10 min with inverted 50 ml beakers on top of each test tube, keep all of the tubes in a boiling water bath for 5 min and then allow them to cool to room temperature.
9. Read the color concentration with a spectrophotometer at 540 nm.
10. Calculate the reducing sugar content of the extracts by reference to a calibration graph plotted from results obtained with glucose: fructose, 1:1 standard solution.
11. Express invertase enzyme activity as mg reduced sugars released g^{-1} soil 24 h^{-1} .
12. To check controls, for assay of invertase activity, make the addition of 1 ml of CaCl_2 solution, 5 ml of the 10% sucrose solution, after the incubation.

3.8 Methods for Phenol Oxidase (Tyrosinase) Enzyme Activity [24–26]

1. Add 0.5 g of soil (<2 mm) into four replicate 10-ml centrifuge tubes (three replicate for samples and one for control).
2. Transfer 3 ml acetate buffer (50 mM, pH 5.0) and rotate on a platform shaker for 10 min.
3. Transfer 2 ml of 10 mM L-3,4-dihydroxy phenylalanine (L-DOPA), to tubes.
4. Rapidly rotate to solution.
5. Put tubes in a shaking incubator (100 rev min^{-1} , 25°C) for 30 min.
6. Promptly centrifuge at 12,000 rpm at 5°C for 5 min to terminate the reaction and filter it.
7. Read the absorbance of the red product in the filtrate with a spectrophotometer at 475 nm.
8. To calculate phenol oxidase activity (μmol product formed dopachrome g^{-1} dry soil h^{-1}), prepare a calibration curve (μmol product formed dopachrome concentration vs. absorbance).
9. To check control, for the assay of phenol oxidase (tyrosinase) activity, make the addition of 2 ml acetate buffer instead of 2 ml of 10 mM L-DOPA.

3.9 Methods for Phosphodiesterase Enzyme Activity [4, 27, 28]

1. Add 1 g of each soil sample (<2 mm) in a 50 ml glass vessel.
2. Transfer 0.2 ml of Toluene, and 4 ml of Tris(hydroxymethyl)-aminomethane (THAM) buffer, pH 8.0, and 1 ml of 0.05 M Bis-*p*-nitrophenyl phosphate (BPNP) solution and rotate the flask for a few seconds to mix the contents.
3. Stopper the flask, bring it in an incubator for 1 h at 37°C for incubation.

4. Transfer 1 ml of 0.5 M CaCl_2 and 4 ml of THAM–NaOH (pH 12) extractant solution, rotate the flask for a few seconds.
5. Filter the suspension.
6. Read the yellow color concentration of the filtrate with a spectrophotometer at 405 nm and calculate as *p*-Nitrophenol ($\mu\text{g pNP g}^{-1} \text{ soil h}^{-1}$).
7. To check controls, for assay of phosphodiesterase activity, make the addition of 1 ml of 0.05 M BPNP solution, after the additions of 1 ml of 0.5 M CaCl_2 and 4 ml of THAM–NaOH (pH 12) solution.

3.10 Methods for Urease Enzymes Activities [4, 27, 29, 30]

1. Add 5 g of moist soil in a volumetric flask (50 ml).
2. Transfer 0.2 ml of toluene and 9 ml THAM buffer and mix the contents.
3. Transfer 1 ml of urea solution and rotate again for a few seconds.
4. Then stopper the flasks and incubate for 2 h at 37 °C.
5. After the incubation, add 35 ml of 2.5 M $\text{KCl-Ag}_2\text{S}_4$ solution, rotate the flask for a few seconds, and stand the solution at room temperature (about 5 min).
6. Complete the contents to 50 ml by addition of $\text{KCl-Ag}_2\text{S}_4$ solution and rotate the contents.
7. Transfer 5 ml of boric acid indicator solution into an Erlenmeyer flask and put it in its special place.
8. Take 20 ml aliquot of the suspension into a distillation flask (100 ml).
9. Add 0.2 g MgO and steam distillate the contents until 30 ml of distillate (~4 min) are collected in the Erlenmeyer flask.
10. Titrate the distillate with 0.005 M H_2S_4 .
11. Read each titrate point, including the control with titration.
12. Calculate the amount of N ($\mu\text{g NH}_4\text{-N g}^{-1} \text{ dwt } 2 \text{ h}^{-1}$) liberated by steam distillation.
13. To check controls, for the assay of urease activity, make the addition of 1 ml of 0.2 M urea solution after the addition of 35 ml of $\text{KCl-Ag}_2\text{S}_4$ solution.

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Part VII

Selection of Microbial Strains for Inoculant Production and Quality Control



Chapter 51

Selection of *Rhizobium* Strain for Inoculum Production

Shreya Desai and Natarajan Amaresan

Abstract

Soil is a hub for the microorganisms from which microbes get the fundamental requirement for their growth and metabolism. Soil also serves as a place for their survival. In turn, microbes also provide the necessary essential requirement for the plant to develop. Microorganisms offer them all the critical nutrients from which nitrogen is the vital nutrient required for the plant to grow. These nitrogen-fixing microbes are known as *Rhizobium*, which gets a place for their survival and gets nutrition. These bacteria also help protect against various microorganisms by producing several secondary metabolites and protecting them from multiple conditions. This relationship between plants and microbes is called symbiosis. Symbiosis opens a new way to find different bio-fertilizers to enhance crop health and productivity by producing *Rhizobium* inoculums.

Key words Symbiosis, Bio-fertilizer, Inoculum, Nutrition

1 Introduction

Bacteria which form the nodule and having healthy relationship with plants are collectively known as Rhizobia. *Rhizobium* is Gram-negative, motile, non-sporulating and rod-shaped bacteria widely distributed worldwide [1]. Rhizobia are the first group of organism realized for its potential of nitrogen fixation. *Rhizobium* fixed atmosphere nitrogen and converted it to ammonia. Ammonia is further used by the plant and supports bacterial growth. These bacteria get nutrition and protected inside the nodule structure with the plants' roots by providing nitrogen. This bacterial association plays a vital role in the growth of plants [2]. In this protocol chapter, *Rhizobium* has been taken as a model organism to select microbial inoculum production. Similar methods also employed for other organisms by changing the growth and nutritional requirements.

2 Materials

1. 70% ethanol.
2. Sodium hypochlorite solution.
3. Yeast extract mannitol agar.
4. Forceps.
5. Nodulated plants roots.
6. Silica gel.
7. Sterile distilled water.
8. 15% glycerol solution.
9. Peat soil, lignite, vermiculite, charcoal, peat, perlite, soil aggregates press mud, farmyard manure, and soil mixture as carrier materials.
10. Yeast extract mannitol broth.
11. Carrier bags.
12. Polythene bags.
13. Adhesives like gum arabic, methyl ethyl cellulose, sucrose solutions, and vegetable oils.

3 Methodology

3.1 Isolation of Rhizobium Strain **[Refer Protocol 1 for Detailed Methods]**

3.1.1 Sampling with Desiccated Silica Gels [3]

1. Excavate whole plants to retrieve root nodules.
2. Carefully remove soil around root nodules.
3. Collect root nodules with forceps.
4. Keep root nodules in a refrigerator at 4 °C until isolation.
5. Collection of root nodules with silica gels prevents decomposition and invasion by soil microbes, affecting the isolation process.

3.1.2 Isolation of Rhizobia from Dried Root Nodules [3]

1. Wash dried root nodules under tap water to remove soil contamination and transfer them to sterile water.
2. Keep the root nodules in sterile water in a refrigerator at 4 °C overnight to absorb moisture.
3. Surface sterilize the root nodules by immersing them in 70% ethanol for 30 s and 3% sodium hypochlorite solution for 3 min.
4. Subsequently, wash root nodules with sterile water five times.
5. Macerate the root nodule in a microfuge tube with 100 µl of 15% glycerol solution.
6. Streak 10 µl of turbid suspension with 15% glycerol solution onto the surface of a yeast extract mannitol agar (YMA)

medium (If no growth, restreak the suspension on YEMA medium).

7. Incubate the plates at 28 °C for 2 weeks in an inverted position.
8. Well-separated single colonies are restreaked onto the fresh plate to obtain pure cultures.

3.1.3 Isolation of *Rhizobia* from the Fresh Nodule

1. Collect roots of legume crops from the field.
2. Clean root nodules, as mentioned above.
3. Cut nodules attached with roots, 2–3 mm on each side of the nodules.
4. Immerse the intact and undamaged nodules in 95% ethanol or isopropanol for 10 s.
5. Transfer nodules and soak for 4–5 min in a 2.5–3% (v/v) solution of sodium hypochlorite or chlorox (commercial bleach) 1:1 (v/v).
6. Rinse the nodules with sterile water 5 times using sterile forceps.
7. Crush the nodule in a sterile tube with a sterile glass rod and sterile water.
8. Streak the slurry on the surface of YMA (Yeast-Mannitol Agar) plates containing congo red.
9. Incubate the inoculated Petri plate at 25–28 °C for 3–10 days.
10. The colony of rhizobia is mucoid, round and shows little or no congo red absorption.

3.2 Rhizobial Inoculant Production

1. Sterilize all the glass vessels to avoid contaminants.
2. Provide sufficient aeration for rhizobial inoculant production.
3. The ideal temperature for rhizobial inoculants production is 28–30 °C.

3.2.1 Points to Remember

1. Sucrose, steep corn liquor, proteolyzed pea husks, and malt sprout extract can be used instead of mannitol.
2. Yeast extract is used as a growth factor supplement for rhizobia.
3. Fresh starch-free cakes of baker's yeast can be used.
4. To produce a high density of rhizobia in the broth, culture can be influenced by culture medium, rhizobial strain, temperature, and aeration.

3.3 Carrier for Bio-fertilizer Production [4, 5]

1. Use peat soil, lignite, vermiculite, charcoal, press mud, farm-yard manure, and soil mixture as carrier materials.
2. For soil inoculation, granular forms of peat, perlite, charcoal, or soil aggregates are suitable.

3.3.1 Points to Remember

A suitable carrier should have the following qualities:

1. Available in sufficient amounts and cheap.
2. Easily available.
3. Provide good adhesion to seeds.
4. Has the adequate buffering capacity.
5. The water-holding capacity of more than 50%.
6. High organic matter content. It should have an excellent water-holding capacity.
7. Enrich soil fertility.
8. Easy to use.
9. Non-toxic to Rhizobia.
10. Easy to sterilize efficiently.

3.3.2 Seed Inoculation [6, 7]

1. Make slurry (bacteria-carrier mixture (neutralize peat soil/lignite for better carrier materials)).
2. Use adhesive to get a tight coating of inoculant on the seed.
3. Prepare seed inoculants by milling the carrier material to a fine powder with a particle size of 10–40 μm .
4. For soil inoculation, use 0.5–1.5 mm granular form of the carrier material.
5. The carrier material is sun-dried up to a moisture level of 5%.
6. Ground the material to the desired fineness.
7. Before sterilization, the carrier is mixed with 10% water.
8. Sterilize the pretreated carrier in an autoclave at 15 lb psi for 3–4 h.

3.4 Production of Carrier-Based Inoculums [8, 9]

1. Aseptically inject the pre-sterilized carrier bag with culture through a syringe fitted with a sterile needle.
2. Disinfect the area of puncture by ethanol.
3. Inject the bacterial culture directly with sterile water into a carrier bag.
4. Immediately seal the puncture hole with the preprinted self-sticking label.
5. For uniform absorption of liquid inoculums, the carrier bags are kneaded by hand or by the shaker.
6. Place the carrier package into a temperature-controlled area.
7. After 2 weeks, the inoculant is ready for use.

3.4.1 Points to Remember

1. The carriers are mixed with finely powdered calcium carbonate to neutralize if they are acidic.
2. Mix CaCO_3 (1%), KH_2PO_4 (0.5%), and soil (25%) thoroughly with charcoal to make it more suitable for the multiplication of rhizobia.

3.5 Curing [10, 11]

1. Keep the mixture of the carrier and the broth for 24 h for curing.
2. Allow the rhizobia to get bind with the carrier within 24 h.

3.6 Packing [12, 13]

1. Pack the inoculants in polythene bags (high density; 0.075–0.090 mm), or polypropylene bags after curing.

3.6.1 Points to Remember

The packing material should have the following properties:

1. Should be stable towards gamma irradiation
2. Should be sterilizable
3. Should have high gas exchange capacity
4. Should not allow high rates of moisture loss
5. Should be non-toxic

3.7 Incubation and Storage

1. Incubate the inoculant for a week with a temperature range of 25–30 °C.
2. Store the packets in a cold room (4–15 °C) until use.

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Mother Culture, Broth, and Peat Test

Dhruti Amin, Sanket Ray, and Vrushali Wagh

Abstract

Biofertilizers are effective microbial technology to overcome ever-increasing global food demand against unfavorable environmental conditions through plant growth promotion and plant protection. The quality of biofertilizers is important criteria resulting in its acceptance by the farmer and an end-user. Certain traits such as purity of cultures (mother cultures), pH, staining, turbidity, total count, and viable count of inoculants must be checked for biofertilizer production and commercialization as a part of a standard procedure.

Key words *Rhizobium*, Biofertilizer, Quality check

1 Introduction

Biofertilizers are the material that consists of living microorganisms like nitrogen-fixing bacteria such as *Rhizobium* spp., *Azotobacter* spp., *Bacillus* spp., *Clostridium* spp., and *Klebsiella* spp.; phosphate solubilizing bacteria (*Pseudomonas* spp., *Bacillus* spp., *Flavobacterium* spp.); and potassium mobilizing bacteria (*Acidithiobacillus* spp., *Bacillus* spp., *Paenibacillus* spp.) [1]. When applied on plants or in the soil, it is responsible for enhancing plant growth, increasing the crop yield, and increasing soil fertility with raise in significant nutrients of the soil like nitrogen, phosphorus, and potassium. There are numerous limitations to biofertilizer use for the agricultural system because their efficacy is not reliable as the biofertilizer mode of action in promoting growth is not well understood. However, research into biofertilizer is increasing, attempting to deal with these issues. Research needs to be conducted to determine how soil type variations, managements, and weather affect biofertilizer efficacy. Quality management is essential and performed continuously to control the microbial products in favor of the customers. The guidelines used for evaluating quality are limited to the available microorganisms density and viability, and

preservation of the guaranteed microorganisms. It is essential to set control plots that do not contain available microorganisms but whose other compositions are the same as the final microbial products. Also, the biofertilizer should manifest the significant effects for quality management of the final biofertilizer products. It is an indispensable requirement to distinguish between the available microorganisms and the supplementary compositions on the impact of the biofertilizer guaranteed by the suppliers. Before the production of biofertilizer by using microorganisms, a mother culture test, broth test, and peat test must be performed to maintain the record. The growth of microorganisms that are used for biofertilizers production must be in pure form. At a regular interval of time, the culture and respective medium (either solid or liquid form) checked for essential morphological and biochemical parameters to maintain maximum growth and function of desired bacteria. These pure mother cultures should be further sub-cultured and held for mass production by accepting standard culture techniques. The pure culture of microbial strains used in biofertilizer production should be maintained in different agricultural universities, regional biofertilizer labs, ICAR, IARI institutions, and NCOF.

2 Materials

Procedures for quality control of biofertilizer.

Quality checks of *Rhizobium* biofertilizer are divided into three parts, i.e., mother culture test, broth culture test, and peat test.

1. Mother/Pure culture of *Rhizobium*.
2. Sterile YEMA agar plate (Table 1).

Table 1

Composition of YEMA medium (Selective media for isolation of *Rhizobium*)

Media constituents	g/l
Mannitol	10.000
Yeast extract	1.000
K ₂ HPO ₄	0.500
K ₂ SO ₄ · 7H ₂ O	0.200
NaCl	0.100
Agar	20.000
Congo red 1% solution	2.5 ml
Distilled water	1000 ml

Table 2
Composition of glucose peptone agar

Media constituents	g/l
Glucose	40.000
Peptone	5.000
Agar	15.000
Distilled water	1000 ml
Final pH (at 25 °C)	7.0 ± 0.2

2.1 Mother Culture Test

2.1.1 Growth of *Rhizobium* on Selective Media Yeast Extract Mannitol Agar (YEMA) [2, 3] (see **Note 1**)

- 1. Inoculating loop.
- 2. Bunsen burner.

2.1.2 Purity Checking of *Rhizobium* Culture

- 1. Mother/pure culture of *Rhizobium*.
- 2. Sterile glucose peptone agar plate (Table 2).
- 1. Inoculating loop.
- 2. Bunsen burner.

2.1.3 Grams Staining

- 1. Mother culture of *Rhizobium*.
- 2. Crystal violet.
- 3. Safranin.
- 4. Gram’s iodine (as a mordent).
- 5. 70% alcohol (as a decolorizer).
- 6. Inoculating loop.
- 7. Bunsen burner.

2.2 Broth Culture

2.2.1 pH

- 1. pH meter.

2.2.2 Staining

Follow materials as mentioned in Subheading 2.1.3.

Grams Staining

Fuchsin Staining

- 1. Ziehl’s carbol fuchsin (Preparation: Dissolve 3.0 g basic fuchsin (C.C.) in 100 ml 95% ethyl alcohol; mix 10 ml with 100 ml 5%

aqueous phenol- made by adding 6 ml 88% liquid phenol to 94 ml H₂O).

2. Slide.
3. Microscope.

2.2.3 Optical Density

1. Broth.
2. Spectrophotometer.

2.2.4 Total Count

1. Petroff-Hausser counter.

2.2.5 Viable Number

1. Culture broth.
2. Spreader.
3. Yeast Mannitol agar + congo red plates.

2.3 Peat Test

2.3.1 pH

1. pH meter.
2. CaCO₃.
3. Inoculant.

2.3.2 Moisture Content

1. Weighing balance.
2. Peat inoculant.

2.3.3 Viable Number

1. Same as Subheading [2.2.5](#).

2.3.4 Plant Infection Method (MPN)

1. Pot.
2. Seed.
3. *Rhizobium* culture.

3 Methods

3.1 Mother Culture Test

3.1.1 Growth of *Rhizobium* on Selective Media YEMA (see **Note 2**)

1. Dissolve weighted quantified media constituents in distilled water. Only dissolve K₂HPO₄ separately. Add distilled water in media to make volume 1000 ml and then autoclave it at 121 °C and 15 lb pressure for 15 min. Sterilize congo red solution separately and add it at the time pouring of media in Petri plates.
2. Streak one loop full of a pure culture of *Rhizobium* on YEMA plates.
3. Incubate the YEMA plates at 26 °C for 10 days. During the incubation period, observe the plates later than 3–4 days and ordinarily after that to develop *Rhizobium* colonies.
4. After incubation, observe large gummy colonies of *Rhizobium* on YEMA plates within 4–5 days. Most of the *Rhizobium* spp.

Table 3
Morphological characteristics of *Rhizobium* sp. on YEMA

Sr. No.	Morphological characteristics	Morphological characteristics of <i>Rhizobium</i> on YEMA
1	Shape	Circular
2	Size	1–3 mm
3	Color	White
4	Opacity	Transparent
5	Margin	Entire
6	Consistency	Moist
7	Elevation	Convex
8	Surface	Smooth
9	Odor	No odor
10	Pigmentation	No pigmentation
11	Special characteristics	Mucoid glistening colonies

strain produce gummy colonies, and some strains produce watery or white colonies on YEMA, as shown in Table 3.

3.1.2 Checking of Purity of *Rhizobium* Culture on Glucose Peptone Agar [4] (see Note 3)

1. Streak one loop full of mother/pure culture of *Rhizobium* on a sterile glucose agar plate.
2. Incubate the plates at 26 °C for 10 days.
3. The appearance of good growth and color changes on glucose peptone agar indicates the presence of contaminants.

3.1.3 Gram's Staining for Mother Culture of *Rhizobium* [5]

1. Prepare a heat-fixed smear of the mother culture of *Rhizobium*.
2. Cover the smear with basic stain crystal violet for 1 min.
3. Add Gram's iodine (mordant) to wash off crystal violet stain and cover the smear with Gram's iodine for 1 min until the smear color turns coffee brown in color.
4. Wash the slide in the presence of a gentle flow of water.
5. Add 70% alcohol as a decolorizing agent in a dropwise manner until the smear's violet color fails to come out from the smear. For decolorization of smear 10–15 s are adequate.
6. Wash the slide with water and observe under oil immersion objective (100×).
7. *Rhizobium* is a Gram-negative, rod-shaped bacteria. Gram's staining is the simplest way to identify the bacteria which belongs to *Rhizobium* spp.

8. Big gummy colonies of *Rhizobium* will appear on the surface of YEMA within 4–5 days of the incubation period. Fast and slow-growing *Rhizobium* will appear in the form of colonies on the surface of YEMA in within 3–5 and 5–7 days, respectively (Table 3).

3.2 Broth Culture [6–9]

Problems with a rhizobial broth culture can often be quickly detected by examining the broth under an adequate microscope.

3.2.1 pH (see Note 4)

1. Monitor broth culture pH at regular time intervals using pH meter.
2. Observe the pH will increase after incubation (for example, pH before growing is 5.0, after incubation, it may become pH 5.1 or more). If broth pH decreases, it means some contaminants occur; lower pH indicates the presence of contaminants.

3.2.2 Staining (see Note 5)

Rhizobial cells are stained by Grams staining and Fuchsin staining for a morphological study like cell shape and size.

Gram's Staining

Follow the procedure, as mentioned in Subheading 3.1.3. Gram-stained cells should appear red, not violet, for rhizobial cells.

Fuchsin Staining

1. Rhizobial cells morphology must be checked routinely using Fuchsin stain.
2. Make a smear of loopful on a clear microscope slide.
3. Fix by brief flaming or drying on a flat surface over boiling water.
4. Stain 30–60 s with Ziehl's carbol fuchsin.
5. Wash in tap water and allow to dry in an inclined position.
6. Note down the observation: Cells of rhizobia are rod-shaped with one or two cells sticking together. They do not appear in long chain. Long-chained cells are indicative of contaminants.

3.2.3 Optical Density (OD) (see Note 6)

Broth turbidity, or optical density using a spectrophotometer.

1. Take 100 µl of broth and measure O.D at 540 nm.
2. It will show 0–1.0 O.D readings.
3. The value of O.D. correlates to the number of cells.
4. If O.D. values are high, then cells number are also high.

3.2.4 Total Count

1. The total count includes viable cells and dead cells by using the Petroff-Hausser counter.
2. At least ten small squares all around the total area to be counted, not only in one large square.

3. Precautions to be taken while performing total cell count, i.e., cells have to be homogeneous, avoid clumping of cells using non-ionic detergent and coverslip must be positioned appropriately to get uniform depth.

3.2.5 Viable Number (see **Note 7**)

1. Count the number of living cells by spread plate or drop plate methods.
2. Take 0.1 ml of sample from 10^{-1} to 10^{-6} or 10^{-7} serial dilutions and spread over on Yeast Mannitol agar + congo red plates in three replicates.
3. Incubate plates at 28–30 °C or at room temperature for 7 days.
4. Take observation: Colonies of rhizobial cells are round, opaque and have smooth margin. They are white and do not absorb red color as well as the other bacteria.
5. Calculation of the number of rhizobia per ml:

$$\text{No. of cells/ml} = \text{no. colonies} \times \text{dilution factor/vol. of inoculum.}$$

3.3 Peat Test

For the peat inoculant, the following quality parameters are checked:

3.3.1 pH

1. Weigh 10 g of inoculant, pour 20 ml of distilled water, mix well with glass rod, incubate at least 30 min, and then measure with pH meter.
2. Maintain neutral pH for the inoculant by adjusting with CaCO_3 (Since peat is acidic, the pH has to be increased with CaCO_3).

3.3.2 Moisture Content

1. To measure fresh and dry weight of peat inoculant.
2. The optimum moisture content of peat inoculant is between 40 and 50%.
3. At low moisture rhizobia will die rapidly. If moisture is high, inoculant may stick to the plastic bag and, thus, not good for rhizobial growth.

3.3.3 Viable Number

The number of viable rhizobia is counted by spread plate method as in the broth test.

3.3.4 Plant Infection Analysis Using Most Probable Number Method (MPN)

1. This is an indirect method of assessing plant infection on nodulation. It is widely used when peat is not sterile.
2. Grow specific plants as per agronomic conditions consider.
3. Infect specific host, i.e., plant with rhizobia.
4. Observe nodulation on the rhizobium treated plant roots. The appearance of root nodule formation is the confirmative evidence for the rhizobium infectivity.

4 Notes

1. The mother culture of the desired strain can be purchased from the microbial culture collection depository.
2. It has to be further sub-cultured and maintained for mass production by adopting standard acceptable laboratory practices under trained microbiologists supervision.
3. Mother culture should be maintained in glycerol stock/slant as a master culture. Its properties such as Gram's nature, its particular traits such as PGPR activity, phosphate solubilization, siderophore production pattern and plant growth hormone production should be monitored regularly to avoid original traits loss.
4. Slow-growing rhizobia of soybeans, mungbean, and peanut produce alkaline compounds which directly or indirectly affects the pH of broth culture.
5. For staining rhizobia, dilute Ziehl's carbol fuchsin with distilled water—1 part dye to 6 parts water by volume. Dilute immediately before using.
6. The optical density method will measure cells from 10^7 to 10^9 per milliliter; if the number of cells is low, this method is not accurate. Moreover, this method has its limitations: (a) It gives a direct count (viable + dead cells), (b) Polysaccharide production in different media gives different results and (c) Limitation from the instrument itself.
7. Viable count analysis of non-sterile peat is more difficult as colonies may sometimes be contaminated by other bacteria. The well-trained staff is needed to conduct this microbiological analysis.

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