

Chapter 2

Collection and Processing for *Trichoderma* Specimen

2.1 Introduction

Fungi are diverse in nature and have been discovered from remote, desert, polar regions, tropical and subtropical regions, and extreme environments. O'Donovan et al. (2016) have been identified about 100,000 fungal species globally. Over the last span, new species of fungi have been identified about 1200 per year. Fungi are commonly eukaryotic microorganisms with imperfect filamentous growth form. A large variety of fungi are collected, isolated, and cultured from soil on the selective or nonselective agar media. Nonselective media are used for isolating a large number of microorganisms in soil. Antibiotics are commonly used, like streptomycin, penicillin, and tetracycline, either alone or in combination. The least selective media isolate may be described approximately 5–15% of the fungal population in soils. Some general observations of fungi have been described by O'Donovan et al. (2016) in the below:

- High C:N ratio media are essential for optimal growth of fungi (low C:N ratio media for optimal growth of bacteria).
- Most fungi are isolated on low pH media (neutral pH media usually bacteria isolated).
- Fungi spores such as *Trichoderma*, *Mucor*, *Aspergillus*, and *Penicillium* in soil grow swiftly on fungal isolation media and prevent the growth of the slower-growing fungi.
- Many fungi produce antibiotic compounds on cultural plates and also inhibit the growth of other microorganisms.
- All types of media are not possible to grow microbes in the agar plates in the laboratory.

Trichoderma species are ubiquitous fungi that are found in all ecosystems, especially in soils microflora. A *Trichoderma*-selective medium (TSM) was established

by Elad et al. (1981) for the isolation of *Trichoderma* propagules from the soil samples. Conversely, Elad and Chet (1983) efficiently isolated *Fusarium* species from the soil using TSM supplemented with benomyl. Captan is additionally added to TSM to inhibit the growth of *Fusarium* species from the media, after that only *Trichoderma* colonies are smoothly grown on TSM.

On Martin's medium (Martin's rose-bengal agar medium), *Trichoderma* isolates grow swifter than other fungi, thus producing larger colonies which can suppress the growth of colonies of other isolates, at the same time reducing CFU (colony forming units) counts. However, many imperfect fungi such as *Aspergillus*, *Penicillium* and soil fungi like *Mucor* spp., *Rhizopus* spp. that grow faster at the same time also inhibit the growth of *Trichoderma* colonies. Moubasher (1965), Mughogho (1968), and Smith and Dawson (1944) used soil extract agar addition to rose-bengal for reckoning soil fungi, including *Trichoderma* species. Recommendation for fast-growing fungi should be marked every day and transferred into new plates. When researchers are used nonselective agar media, i.e., PDA, *Trichoderma* colonies are difficult counted in soil samples because of other fungi/or microbes rapidly overlapping grown. Elad and his research team (1981) used fenaminosulf for suppressing oomycetes, at the same time to enhance the growth and sporulation of *Trichoderma* colonies. They reported that an addition of metalaxyl or propamocarb inhibited *Pythium* growth on the medium. The colony of *Aspergillus* is usually lower on TSM + P (propamocarb), while *Penicillium* colony is generally higher on TSM + M (metalaxyl). *Aspergillus* and *Penicillium* spp. indicated a slight suppression by propamocarb during incubation temperatures of 30 °C (Elad et al. 1981).

A *Trichoderma*-selective agar medium (TSM) mainly selects based on two criteria: (1) *Trichoderma* colonies have high tolerant levels of pentachloronitrobenzene (PCNB) and rose bengal; and (2) they can grow and sporulate at a low concentration of glucose on media. The media uses chloramphenicol for antibacterial activities, and pentachloronitrobenzene, p-dimethylaminobenzenediazosodium sulfonate, and rose-bengal used for inhibition of specific fungal activities. The addition of metalaxyl or propamocarb or dexton does not inhibit the growth of *Trichoderma* colonies on the media. In the presence of Dexton is assisted faster growing of *Trichoderma* colonies and freely produced typical green colour, which supported in the identifications among other soil-borne fungi. The main objective of this chapter, the use of established method of TSM is more effective isolated of *Trichoderma* propagules in naturally infested and artificially inoculated soils.

2.2 Media for Isolating of *Trichoderma*

2.2.1 *Trichoderma* Selective Media

Trichoderma-selective medium (TSM) is established for quantitative isolation of *Trichoderma* propagules from diverse soil flora. Chloramphenicol used as a bacterial inhibitor. The selective fungal inhibitors commonly used as

p-dimethylaminobenzenediazo sodium sulfonate, pentachloronitrobenzene, and rose-bengal. A low concentration of glucose contains on TSM which allowing rapid growth of *Trichoderma* colonies or sporulation. *Trichoderma* selective media consisted in the below components:

1. 0.2 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$
2. 0.9 g of K_2HPO_4
3. 0.15 g of KCl
4. 1.0 g of NH_4NO_3
5. 3.0 g of glucose
6. 0.15 g of rose bengal
7. 20 g of agar
8. 0.25 g of chloramphenicol
9. 0.3 g of *p*-dimethylaminobenzenediazo sodium sulfonate
10. 0.2 g of pentachloronitrobenzene

The above all chemicals are dissolved in 1000 mL of distilled water of Erlehnmeier flask, stirred, warm to boil in oven up to cleared, with cotton wool placed over the mouths except chloramphenicol and pentachloronitrobenzene. The flask is autoclaved at 121 °C, 1.4 kg cm⁻¹ for 15 min after autoclave added respectively 0.25 g chloramphenicol and 0.2 g pentachloronitrobenzene into solution. After that TSM medium incubated at 45 °C to prevent the media solidification.

2.2.2 *T. harzianum*-Selective Medium (THSM)

This medium assists as comparisons study of aggressive (*Agaricus bisporus*) with nonaggressive *T. harzianum* groups. The medium included some antimicrobials compounds such as streptomycin, propamocarb, chloramphenicol, and quitozene which are highly selective and allowing the colony growth of *T. harzianum*, with absence of visible microbial contaminants. The THSM consisted in the below components (Williams et al. 2003):

1. 0.2 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$
2. 0.9 g of K_2HPO_4
3. 1.0 g of NH_4NO_3
4. 0.15 g of KCl
5. 0.15 g of Rose Bengal
6. 3 g of glucose
7. 20 g of agar
8. 950 mL of distilled water

The above medium in conical flask is autoclaved at 121 °C, 1.4 kg cm⁻¹ for 15 min. After autoclave, 0.25 g of chloramphenicol (per litre, antimicrobial), 9.0 mL of streptomycin (fungicide) (1% wt/vol of stock solution), 1.2 mL of propamocarb (772 g of active ingredient per litre) and 0.2 g of quitozene, all in 40 mL of sterile distilled water, and the mixture is added to the cooled basal medium.

2.2.3 *Rose Bengal Agar (RBA)*

Fungi commonly discovered from soil, lakes, air, ponds, rivers, streams, wastewaters, polar-regions (Antarctica), tropical and subtropical regions, well waters and elsewhere. Naturally fungi is heterotrophic, they have ability to adapt diverse biodiversity conditions. Fungi are normally contaminated in various commodities including food and beverage products, food storage facilities, and food processing equipment's. Yeasts and moulds can initiate growth over wide ranges of pH and temperature in almost all types of food such as food ingredients and foods processes. RBA commonly used for the isolation of fungal colonies from diverse biodiversity. RBA media comprises the following components (Madigan et al. 2000):

1. 1.0 g of KH_2PO_4
2. 0.5 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$
3. 5.0 g of peptone
4. 10.0 g of dextrose
5. 0.35 g of Rose Bengal
6. 1000 mL distilled water

The above medium in conical flask is autoclaved at 121°C , 1.4 kg cm^{-1} for 15 min, after autoclave added 0.1 g of streptomycin sulfate or 0.25 g of chloramphenicol. Streptomycin sulfate/chloramphenicol usually used to inhibit the growth of bacteria. After that RBA medium incubated at 45°C to prevent the mixture solidification or directly use for further experimental works. In addition, rose bengal and streptomycin sulfate/chloramphenicol are added in the media to enhance the selectivity and to control the overgrowth by faster growing moulds (i.e., *Neurospora*, *Rhizopus*). Smith and Dawson (1944) mentioned that a near-neutral medium (pH of 6.8) added to Rose Bengal, because more colonies grown than acidic medium (pH of 4.2). RBA uses peptone as a source of carbon and nitrogen, respectively dextrose as energy source, and MgSO_4 to provide trace elements.

2.3 Materials

1. Soil/sediment/mud.
2. Balance.
3. Hot water bath (45°C).
4. Microwave ovens.
5. Blue screw bottles and test tubes.
6. Sterile pipettes and tips.
7. Vortexes.
8. Spreaders for spread-plating (glass pipette).
9. Squirt bottles and dishes (70% ethanol).
10. Bunsen burner.

11. Loops and needles.
12. Conical flasks (several volumes).
13. Cotton wool/muslin cloth.
14. Petri dish (plastic/glass).
15. Tubular augur (length 2.5 m and diameter 1.9 cm).
16. Sterilized blade or knife.
17. Sterilized containers.
18. Incubator.
19. Chillier.
20. Freezer.
21. Universal bottle.

2.4 Methods

2.4.1 *Sample Collection Procedures*

1. Soil samples can be collected from four cardinal points per reference/selected crops (plant) during different sampling periods (at an interval of 45 days between each sampling period; depend which type of plants or crops) as shown Fig. 2.1.
2. The soil samples should be taken out from a depth of 10–15 cm with horizontal distances of 100–120 cm.
3. A custom-made soil augur (length 2.5 m and diameter 1.9 cm) is pushed into the soil up to approximately 10 cm deep and then pulled out.
4. A steel handle can be used to push out the soil core and separate it out from the tubular augur with a sterilized blade or knife.
5. From this soil core, take approximately 400 g of samples and place into sterile containers/polyethylene bags, cover with lids/tight with wire and label with the information of the collection sites, date, and origin of the samples.
6. Measure the pH range of the soil samples.
7. Bring the collected soil samples to the laboratory as soon as possible for analysis or store at 4 °C until further use.

2.4.2 *Isolation of Trichoderma Colony*

Populations of the indigenous *Trichoderma* colonies are counted by using soil serial dilution plating and on the basis of colony forming unit (CFU). This technique is very simple and easy, low cost and is suitable for handling a large quantity of samples within a short period.

Fig. 2.1 Soil sample collection from plant



2.4.3 Sample Dilution

1. Ten grams (10 g) of soil sample is weighed out and placed into a sterile conical flask, then added 100 mL sterilized distilled water with 0.1% (w/v) of Bacto Agar (Difco, USA) sterilized at 121 °C/1.05 kg/m² for 15 min.
2. Place the conical flask on the rotary MaxQ Mini 4450 shaker for shaking at 210 rpm for 20 min.
3. Keep the conical flask in the laminar flow for at least 10 min.
4. Transfer 1 mL of soil dilution into a 15 mL falcon tube containing 9 mL of sterile distilled water using a sterilized pipette. It can create a 1/10 dilution (10^{-1}) of the sample (as shown Fig. 2.2). The solution of falcon tube is vortex for at least 1 min or wait until the sample properly mixed. Ensure that the sample is labeled appropriately.
5. Again transfer 1 mL of the 1/10 dilution into a 15 mL falcon tube, adding 9 mL of sterilized distilled water using a sterile pipette. It can create a 1/100 dilution (10^{-2}) of the sample. The sample solution of falcon tube is homogeneously mixed by vortex and labeled properly with an appropriate dilution.
6. The above steps (4–5) again repeat to prepare additional dilutions if necessary. Usually, serial dilution of 10^{-3} is more appropriate to count the growth colonies of *Trichoderma* using CFU method. Fungal cultures normally not exceed a 1/10,000 of dilutions.

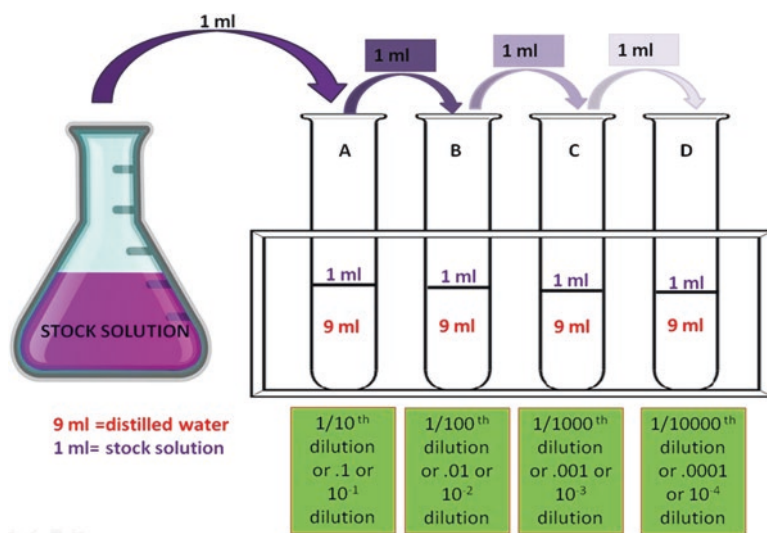


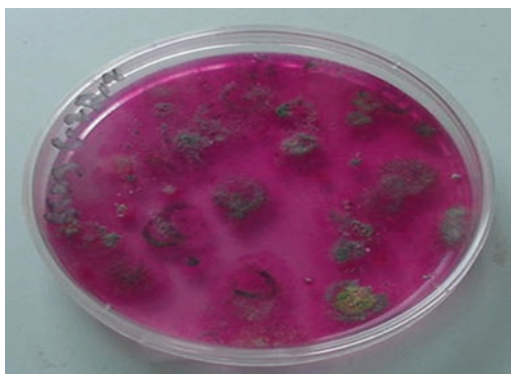
Fig. 2.2 Procedures of Serial dilution in microbiology

7. The specific serial dilution is selected based on the growth of *Trichoderma* colonies existed in the collection site. Population densities are commonly measured according to the serial dilution number.

2.4.4 Culturing Plates

1. Transfer 1 mL of suitable diluted samples or appropriate sample dilution using a sterilized pipette (if do not know suitable dilution, then several dilutions need to be plated out) and placed each petri dish, after that add 9 mL of the selected media (TSM/THSM/RBA).
2. The spreader (bent glass rod) which should be flame-sterilized, to spread (cool the spreader before spreading) the dilution over the petri dish surface and make sure the dilution properly distributed, and then keep the plate for solidification.
3. Glass rod should be flame-sterilized between different dilution factors onto media. The moisture should be allowed to absorb into the agar before incubation.
4. Label the plates with date, serial dilution factor, and incubation temperature (28 ± 2 °C). Each dilution should prepare triplicate plates.
5. The cultural plates should incubate at the specific temperature of 28 ± 2 °C and scrutinize the plate daily until day 7 for the estimation of *Trichoderma* colonies in the soil samples (as shown Fig. 2.3). Very slow growing of fungi colonies should not visible until day 7. Each single colony is scored as a Colony Forming Unit (CFU).

Fig. 2.3 Seven-day old culture showing *Trichoderma* CFU on TSM



6. After *Trichoderma* colonies are readily sporulated (2–7 days) in agar plate, the number of colonies are counted on each plate and calculated the CFU in soil samples (not longer than 24 h store the plates at 4 °C before counting if needed).
7. All accounted colonies are individually cultured onto fresh PDA (Difco: USA) plate.

2.4.5 Preparation of Slant Media

1. About 20.0 g of dextrose, 4.0 g of potato starch, and 15.0 g of agar is dissolved in 1000 mL of distilled water of Erlehnmeier flask, stirred, warm boiled into oven and cotton wool placed as stoppers at the mouths, then autoclaved the flask at 121 °C, 1.05 kg/m² for 15 min.
2. After autoclave, adding 25.0 mg of chloramphenicol or 0.1 g of streptomycin sulfate. Chloramphenicol/streptomycin sulfate used to inhibit any bacterial growth of competing microorganisms from mixed specimens.
3. Remove caps/covers of sterile universal bottles/tubes, after that the bottle/tube mouth sterilized by flame several times.
4. Later 2–3 mL of molten media is added to the bottles/tubes using sterilized pipettes. Do not lay down or allow to touch anything the caps/covers or pipettes.
5. Replace the caps/covers of the bottles/tubes at 45° angle until the molten media solidification.
6. The molten agar is never permitted to get in contact with the caps/covers during slanting (Fig. 2.4).

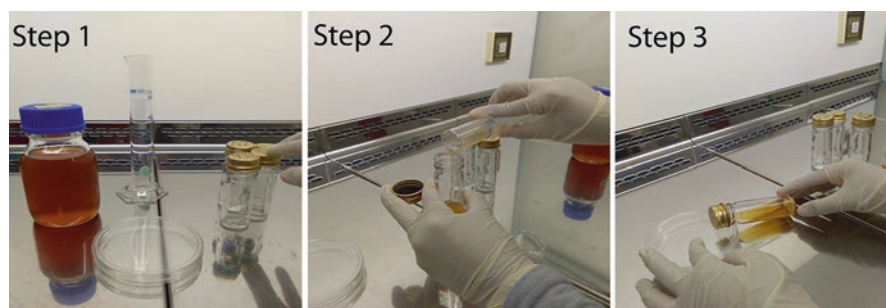


Fig. 2.4 Step by step preparation of slant

2.4.6 Slant Culture

1. Remove the cap/cover of slant bottle/tube (hold the bottle/tube in your hand), flame the tops layer several times, and transfer the culture from the edge site of day three (3) culture of isolates. Use the inoculating loop. Remember to sterilize it before and after each use. Inoculate at least two slants culture for each isolate.
2. Inoculate your slant by moving the loop gently up of the surface of the agar. If there is any liquid in the bottom of the slant bottles/tubes avoid sticking the loop into this condensate.
3. Incubate the slant culture under incubator chamber temperature of 28 ± 2 °C. The cap/cover should be loosed when incubated to allow gas exchange.
4. Label it with: date and isolation name. The label should be marked so that it will not affect the observations of growth of *Trichoderma* colony.
5. Observe at least once daily until completely grown into slant.
6. Keep the slant culture at 4–10 °C until necessary for further uses.

2.5 Identification of *Trichoderma* Isolates

The estimation of *Trichoderma* colonies are identified to the genus or species level using conventional techniques based on the macroscopic characteristics (e.g., colour, odour, physiological characters, growth rate) and microscopic characteristics (e.g., mycelium, conidia, conidiophore, phialides and chlamydospores). Conventional techniques based identification of *Trichoderma* isolates are detail described in [Chap. 4](#).

2.6 Pure Culture of *Trichoderma* Isolates

Pure culture of *Trichoderma* technique is commonly used by dilution series. This technique concept becomes more popular and comfortable. A dilution series make a fungal isolate that sporulates abundantly grow per agar plate maximum five fungal spores. Cultural plate should add 10 mL sterile distilled water to prepare spore suspension. The spore suspensions have millions of spores per mL. The number of spores is calculated per mL using hemacytometer; after serial dilution approximately 5 spores can place onto agar medium plus 250 µg/mL of chloramphenicol/streptomycin as a bacteria inhibitor. For example, cultural suspension tube that contains 1×10^6 spores per mL then dilutes the mix 100-fold (10^{-2}), the concentration is now 1×10^{-4} /mL. Again dilute this mix by 100-fold (current dilution of 10^{-4} or 10,000-fold dilution from the original concentration), the final concentration reach 102 spores per mL or 100 per mL. If 1 mL of this dilution is added per plate, it will reach 100 spores. If you follow this procedure in the lab, you will get the known amount of dilute spores per plate in order to 50, 100, or 200 spores/plate.

2.7 Direct Culture Methods

It is possible to directly inoculate of *Trichoderma* colony to directly place soil samples onto the selective agar (TSM, THSM, and RBA) media: directly small portion of soil samples placed on agar plate then incubated at temperature of 28 ± 2 °C. The growing colonies are transferred onto fresh PDA plates and incubate it to the steady growth of *Trichoderma* colony.

2.8 Storage of *Trichoderma*

Continue subcultures of *Trichoderma* isolates are expensive and also difficult to maintain the pure culture. Another problem continuous subculturing of isolates may mutate and slowly reduce the metabolic rate. Preservation of isolates can be carried out by several ways as described below:

2.8.1 Storage in Water

Pure cultures of *Trichoderma* are kept in universal bottle covering sterilized water. After that bottles are stored at 7–10 °C until 4–5 years. This way of preservation of *Trichoderma* isolates is simpler and easier.

2.8.2 Cold Temperature

The cultural *Trichoderma* slants (plates) are kept at 45 °C angle and stored above 4 years at cold temperatures of 4–10 °C.

2.8.3 Under Mineral Oil

Trichoderma isolates are cultured onto low concentration of sugar media in universal bottles. These universal bottles should allow to entirely growing the agar surface. The cultural agar is absolutely covered with sterile mineral oil. The cultural universal bottles can be stored at room temperature for 1 year. Because, the cultures are taken oxygen slowly from oil. The culture covered all times by at least 1 cm of mineral oil and replaced the sterile lids; otherwise the top of the bottles can allow the growth of unknown fungi because it has nutrients. If bottle lid/cover can loose by room air pressure so air can easily movement (in and out) in bottle. So the author is recommended that covering the oil should be below the lid. Before subculturing, it is essential to sterile the surfaces of bottle lids by flaming.

2.8.4 Freeze Drying or Lyophilization

The cultural isolates are placed in a lyophilization tube and then cooled and freeze-dried. After that it can be stored for more than 20 years. This storage process is commonly used only for spore-forming imperfect filamentous fungi (Deuteromycetes), but nonspore-forming fungi are less successful.

2.8.5 Low-Temperature Storage

Trichoderma slants are submerged in 10–25% sterile glycerol in water and then sealed. Later it can be stored at –70 °C or kept in liquid nitrogen for long-term preservation.

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