Fungal Biology

Shafiquzzaman Siddiquee

Practical Handbook of the Biology and Molecular Diversity of *Trichoderma* Species from Tropical Regions

Springer

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Series Editors

Vijai Kumar Gupta
School of Natural Sciences
National University of Ireland Galway
Galway
Ireland

Maria G. Tuohy
School of Natural Sciences
National University of Ireland Galway
Galway
Ireland

Fungal biology has an integral role to play in the development of the biotechnology and biomedical sectors. It has become a subject of increasing importance as new fungi and their associated biomolecules are identified. The interaction between fungi and their environment is central to many natural processes that occur in the biosphere. The hosts and habitats of these eukaryotic microorganisms are very diverse; fungi are present in every ecosystem on Earth. The fungal kingdom is equally diverse, consisting of seven different known phyla. Yet detailed knowledge is limited to relatively few species. The relationship between fungi and humans has been characterized by the juxtaposed viewpoints of fungi as infectious agents of much dread and their exploitation as highly versatile systems for a range of economically important biotechnological applications. Understanding the biology of different fungi in diverse ecosystems as well as their interactions with living and non-living is essential to underpin effective and innovative technological developments. This series will provide a detailed compendium of methods and information used to investigate different aspects of mycology, including fungal biology and biochemistry, genetics, phylogenetics, genomics, proteomics, molecular enzymology, and biotechnological applications in a manner that reflects the many recent developments of relevance to researchers and scientists investigating the Kingdom Fungi. Rapid screening techniques based on screening specific regions in the DNA of fungi have been used in species comparison and identification, and are now being extended across fungal phyla. The majorities of fungi are multicellular eukaryotic systems and therefore may be excellent model systems by which to answer fundamental biological questions. A greater understanding of the cell biology of these versatile eukaryotes will underpin efforts to engineer certain fungal species to provide novel cell factories for production of proteins for pharmaceutical applications. Renewed interest in all aspects of the biology and biotechnology of fungi may also enable the development of "one pot" microbial cell factories to meet consumer energy needs in the 21st century. To realize this potential and to truly understand the diversity and biology of these eukaryotes, continued development of scientific tools and techniques is essential. As a professional reference, this series will be very helpful to all people who work with fungi and should be useful both to academic institutions and research teams, as well as to teachers, and graduate and postgraduate students with its information on the continuous developments in fungal biology with the publication of each volume.

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Shafiquzzaman Siddiquee
Biotechnology Research Institute
Universiti Malaysia Sabah
Kota Kinabalu, Malaysia

ISSN 2198-7777 ISSN 2198-7785 (electronic)
Fungal Biology
ISBN 978-3-319-64945-0 ISBN 978-3-319-64946-7 (eBook)
DOI 10.1007/978-3-319-64946-7

Library of Congress Control Number: 2017949181

Printed on acid-free paper

This Springer imprint is published by Springer Nature
The registered company is Springer International Publishing AG
The registered company address is: Gewerbestrasse 11, 6330 Cham, Switzerland

Preface

Almost 223 years ago in 1794, the generic name of *Trichoderma* was first introduced by Persoon based on material collected from Germany. *Trichoderma* are fungi ubiquitous in all ecosystems; they are known for their rapid growth and capacity in utilising diverse substrates and are relatively easy to isolate and culture. Over the past decade, they have become a very important resource for the biotechnology industry because of both their ability to secrete large amounts of cellulolytic and hemicellulolytic enzymes and the biocontrol capabilities in some of its species against nematodes, insects, and phytopathogens.

Most of the *Trichoderma* species grow rapidly in artificial culture and produce large numbers of small green or white conidia from conidiogenous cells located at the ends of conidiophores. Morphology characters are variable to a certain degree in their color, shape of conidia, conidiophore, pustules, phialade, and chlamydospores. These characters allow a comparatively easy means for identifying *Trichoderma* as a genus, but the species concept is difficult to deduce and there is considerable confusion over the application of specific names. The taxonomy of *Trichoderma* is rather difficult and complex due to the plasticity of characters based on classical approaches. Molecular tools have revolutionised systematic and phylogenetic research and are now routinely used in systematic laboratories. Most journals publishing molecular phylogenies required the sequences to be deposited in accessible repositories. However, as more molecular data becomes available, there is a growing concern about the taxonomic origin of this data. Although some taxa appear to be well outlined and easy to recognise, others can only be identified by a handful of specialists. Nowadays, there are a large number of sequences deposited in GenBank that are incorrectly labeled and, unless remedied, these will continuously be associated with the wrong taxa. In my opinion, an essential link between data and taxa can provide a means to verify the taxonomic characters of the isolates sequenced, and macroscopic and microscopic characteristics. Otherwise, a species level identification study cannot be corrected or uncorrected and the user has to rely on the person making the misidentification.

For this situation, the Practical Handbook has described a right pathway to solve the controversial identifications of twelve (12) *Trichoderma* species and also provide clear in-depth information in each chapter—introducing the basic concept of microbiology, collection and processing for *Trichoderma*, slide culture techniques, macroscopic and microscopic analysis, and molecular tools using detailed photographs or drawings of the strains. It is easy to ensure and recognise the quality of the results, even though it is possible to go back to the source of the information. In conclusion from the above discussions, the Practical Handbook has entirely described the combination of macroscopic and microscopic characteristics, and molecular data that are undoubtedly identified at the species level as a paradigm reference for the accurate identification of *Trichoderma* species.

Although this Practical Handbook will be of use to anyone interested in the subject matter, it will be of particular benefit to specialized microbiologists/researchers/lab technician as well as those who simply use microbiology as an adjunct to their own discipline, in finding relevant information quickly and easily.

The author is grateful that Springer Nature has enthusiastically agreed to publish this work and appreciates their help and collaboration on this project. Lastly the author would also like to acknowledge Dr. Vijai Kumar Gupta. He is the one without whom this journey would never have started and also never have come to an end.

Kota Kinabalu, Malaysia Shafiquzzaman Siddiquee

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About the Author

Shafiquzzaman Siddiquee, Ph.D., is an associate professor at the Biotechnology Research Institute of Universiti Malaysia Sabah (UMS), Malaysia. Dr. Siddiquee obtained his M.Sc. (Molecular Microbiology) and Ph.D. (Bioelectrochemistry) degrees in 2008 and 2010, respectively, from University Putra Malaysia (UPM), Malaysia. In 2011, Dr. Siddiquee received a **Best Post Graduate Award** from UPM for being the most outstanding postgraduate student of the year. In 2014, he was the winner of the **ProSPER.net-Scopus Young Scientist Award** in the category of Sustainable Agriculture. He served as a guest professor at the Institute of Analytical Chemistry, Chemo- and Biosensors of the University of Regensburg, Germany. Currently he is a head of the UMS Biosensor Research Group. He is involved in varying types of research areas, including chemo- and bio-sensors based research, and *Trichoderma* biodiversity and its applications. He has published over 76 refereed journal articles, 4 books, 10 book chapters, and 30 proceeding papers. His findings yielded, so far, 4 patents, 5 invited speaking engagements, and 40 research awards, including awards from the 2015 MOHE entrepreneurial Project Award and the International Conference and Exposition on Inventions by Institutions of Higher Learning 2015 (PECIPTA). Both nationally and internationally, Dr. Siddiquee has established relevant research group networks. In pop culture, Dr. Siddiquee's findings have been broadcast in Malaysian national television (TV1, TV2, and TV7) and highlighted in various print media.

Chapter 1
The Basic Concept of Microbiology

1.1 Aseptic Technique and Safety

Aseptic technique is a method that involves target-specific practices and procedures under suitably controlled conditions to reduce the contamination from microbes. It is a compulsory laboratory skill to conduct research related in the field of microbiology. Mycologist/microbiologists must follow aseptic techniques for multiplicity procedures such as screening of isolates/strains, pure cultures, slant cultures, single spore cultures, microbes transferring cultures, inoculating media, and conducting several microbiological experiments. Proper aseptic technique has prevented the cultures contamination from inborn and outborn microbes in the environment. As example, airborne microbes (e.g., fungi) handpicked from the surveyor's health, the lab benchtop, unsterilized glassware and equipment, dust, and other areas, thus interfering to get proper experiment results. Using the proper aseptic technique can significantly reduce/minimize the risk of contamination. Aseptic technique commonly maintains pure stock cultures and single spore culture while transferring cultures into fresh media. Appropriate aseptic techniques prevent microbes from unintentionally released into the environment and/or contaminating lab user in the laboratory. Aseptic techniques have the following objectives: (1) to acquire the knowledge of aseptic technique in the field of microbes, (2) to avoid the contamination of cultures from undesirable microbes in the laboratory, (3) to subculture (transfer cultures from one media by inoculating into another media), (4) to isolate pure culture from mixed culture, and (5) to inhibit lab microbes from being distributed in the environment and/or contaminating the investigator (vlab.amrita.edu, 2011).

Biosafety comprises addressing of the safe handling and containment of transferable microorganisms and unwarranted biomaterials. The basics of containments are safety equipment, microbiological practices, and skill safeguards that defend lab workers, environment, and examiners/public from exposure to transferable microbes

S. Siddiquee, *Practical Handbook of the Biology and Molecular Diversity of Trichoderma Species from Tropical Regions*, Fungal Biology,
DOI 10.1007/978-3-319-64946-7_1

that are properly handled and kept in the laboratory. Personnel working with transferable agents are acquainted with hazards that require the proper training and skillful practices for handling such materials. All laboratories are required to develop a biosafety or operations manual that categorizes the hazards that may be met, and that specifies the proper practices and techniques to reduce/or minimize the hazard exposures. A microbiologist/scientist, and knowledgeable laboratory techniques, safety procedures, and hazards associated with handling transferrable agents will accountable for the comportment of research with infectious agents or materials. The proper design and work structures, safety equipment, and controlling practices should enhance the laboratory personnel and safety practices.

Cliffe (2016) has mentioned that many biosafety levels (BSLs) have been developed for laboratories to enhance the protection levels of environment and staff. BSLs have standard guidelines that described the proper containment equipment, services, and procedures for apply by laboratory researchers/scientists/technicians/students. The BSLs are categorized into BSL 1 to BSL 4, and the risk associated with every BSL increases with the infectious microbes encountered. BSL 2 practices are mostly followed by clinical microbiology laboratories. When conducting works with high transmissible agents, the risk of aerosol transmission is so high, so microbiology laboratories must follow BSL 3 practices.

Hint

Risk group	Pathogenicity features
BSL 1 (low individual and community risk)	Implausible to cause significantly human disease or animal disease of veterinary. *E. coli*, *Pseudomonas, Baculovirus* of insects, *Aspergillus*
BSL 2 (moderate individual risk, limited community, livestock or environment risk)	Causing human or animal disease but noncontaminant to laboratory researchers, technicians, communities, livestock, or environment. Infectious risk is *via* direct contact, ingestion, or inhalation. Effective treatment, preventive and control measures, are readily available. *Bacillus cereus*, Salmonella, Shigella, Hepatitis A, B, C, Rubella, Chikungunya
BSL 3 (high individual, low community risk)	Exotic or indigenous agent—potentially transmit disease mainly via aerosols. Disease caused is severe and can kill. Low risk to community—effective treatment, preventive available: SARS, Yellow fever virus, H5N1, Rabies virus, BSE, *Bacillus anthracis*, TB, Rickettsia
BSL 4 (high individual and community risk)	Causing life-threatening human disease. Disease transmissible—one individual organism to another organisms—via aerosol or unknown. No available effective treatment, preventive and control measures. Ebola virus, Herpes B virus, Smallpox virus

In Biosafety Level 1 (BSL-1) denotes a fundamental level of containment that be contingent on standard microbiology practices with never requiring any special primary or secondary barriers recommendation, other than washing hand. BSL-2 practices are appropriate to teaching, diagnostic, clinical, and other laboratories in which work has conducted wide-range of indigenous moderate-risk agents that are available in the community and allied with human disease.

The objective of this chapter is to provide the proper hand-on practices in microbiology laboratory to teachers, students, researchers, scientists, and technicians and also ensure the studies proceed safely and succeed the successfully of educational standard.

1.2 Good Microbiological Laboratory Practices (GMLP)

Good microbiological laboratory practices (e.g., aseptic technique) when handling microorganisms have prevented contamination of the workplace from other microorganisms or nontarget microorganisms. They supplement the containment facilities, procedures and processes to reduce the spread of microorganism contamination and prevent the exposure of people and the environment to the microorganisms that are being deliberately manipulated either by accident or once work has finished.

The principles of GMLP summarized below should be adopted when working with microorganisms, so it should be declaim in conjunction with the guidance on containment.

Principles of Good Microbiological Practices (Chosewood and Wilson 2009)

1. **Never** uses any pipette substance by mouth. In laboratory always avoid contact hand to mouth or hand to eye. In the laboratory, never drink, eat, apply cosmetics, lip balm, handle contact lenses and taken medication.
2. Aseptic techniques properly **use**. Right way wash your hand after removing gloves, other personnel protective equipment (PPE), handling potentially infectious agents or materials and exiting of laboratory.
3. Centers for Disease Control and Prevention (CDC)/National Institutes of Health (NIH) *Biosafety in Microbiological and Biomedical Laboratories* (BMBL) have recommends that laboratory personnel must be protected their streetwear clothing from contamination by wearing appropriate attire (e.g., gloves, lab shoes or use shoe covers) when conducting works in BSL-2 laboratory. Streetwear clothing and street shoes are not permitted for use in BSL-3 laboratory; in the lab always preferred to change of streetwear clothes and shoe covers or shoes keen for uses. In BSL-4 is mandatory changed from streetwear clothes/shoes to use the permitted laboratory attires and footwear.
4. Once removing the hazardous material in the laboratory, personnel must be followed Occupational Safety and Health Act (OSHA). When infectious agents are handled in laboratories; syringes, and needles or other sharp instru-

ments should be restricted. A used needle **never** repetited. Dispose of syringe–needle assemblies in properly labeled, lesion resistant, autoclavable sharps containers.

5. Knob infectious materials as determined by a risk assessment. Airborne infectious agents should be controlled by a certified biosafety cabinet (BSC) appropriate to the BSLs and risks for the agent.
6. Make sure that engineering controls (e.g., eyewash, BSCs, safety showers, and sinks) are properly maintained and inspected.
7. The contaminated lab ware or materials **never** leave open to the outside of BSC. All biohazardous materials steadily stored with proper labeled, and sealed containers.
8. Must be displayed all laboratories doors with the recognized biohazard symbol, a list of the infectious agents, access requirements (e.g., PPE) and emergency contact information.
9. Avoid the use of aerosol-producing techniques when working with infectious materials. Pipette mixing, needle clipping, centrifugation, and sonication can generate considerable aerosols.
10. Antiseptic traps and inline filters on vacuum lines are used to defend vacuum lines from contamination.
11. Follow the laboratory biosafety design for the infectious materials that are conducting with the accurate decontamination methods to decontaminate the infectious materials. **Always** keep spill kits available in the lab for managing an accidental spill of pathogenic materials.
12. Clean the worktable area with an appropriate disinfectant after conducting work with infectious materials in the laboratory. **Must not** be cluttered the laboratory containment in order to authorize suitable floor and disinfect work area.
13. Without decontaminated or sterilized or autoclaved (temperature (121 °C), pressure (15 psi), and time (15–20 min)), **never** allow to leave unwanted infectious materials or contaminated agents in the laboratory or to be put in the sanitary sewer.
14. When transferring or shipping infectious materials to other laboratory, **always** use Postal or Department of Transportation (DOT) approved, leak-proof sealed and properly packed containers (primary and secondary containers). Be sure the lid is on tight and avoiding the container contamination from the outside. Before transporting infectious materials, make sure that the outside of container is decontaminated. Ship infectious materials in accordance with Federal and local necessities.
15. All accidents, occurrences, and unexplained illnesses **must be** reported to lab technician/supervisor and the Occupational Health Physician.
16. **Always** think safety first priority during laboratory operations. Evoke, if you are not clearly apprehend the proper handling and safety procedures or how properly use safety equipment; avoid conducting work with the infectious materials until you get proper instruction or consult the CDC/NIH BMBL for further information.

(*Note: Remember, the abovesaid principles of good microbiological practices will help and protect you, your fellow worker and the public from the infectious agents you use.*)

1.3 Risk Assessment

Teachers, researchers, scientists, and lab technicians may make practical amendments to the risk assessments model according to their professional judgment based on their proficiencies. The legislation governing Hazardous Substances and Dangerous Goods entails that entire procedures and experiments involving hazardous materials must have documentation and minimize exposure. The risk management approach always apply for safety in the laboratory before starting any new research projects or experiments work. Conducting risk assessment it should detect potential hazards and control the actions to reduce any risks of personnel health.

Factors to be considered in risk assessments (Source: ASE 2001).

Factor	Relevance
Good microbiological laboratory practice (GMLP)	Protection of operators (teachers, researchers, scientists, students, and lab technicians)
Levels of practical works (levels 1, 2, 3, and 4)	Degree of risk of microorganisms culture; well-trained of teacher and technician; student age and discipline
Select the microorganisms (ACDP hazard group 1)	Cultures must present minimum risk when GMLP are followed
Source of cultures	Reliable and professional supplier or permitted environment samples
Type of investigations/activities	Ample containment of cultures; hands-on work
Composition of culture media	The uses of some culture media are planned for selecting the growth of pathogens, i.e., not in ACDP hazard group 1
Volume of cultures	Increased risk with increase in volume of liquid culture
Laboratory facilities	Suitable level of containment for hands-on microbiological work
Equipment	Adequate for purposes
Incubation conditions	Proper selecting for the growth of pathogens
Disposal of contaminated materials	Ensures the elimination of risk to others
Proficiency of lab technicians, researchers and teachers	Skill and proper training in practices and procedures applicable to the level of practical work (levels 1, 2, 3, and 4)
Student age and discipline	Suitable level of practical work; self-confidence in class discipline
Sources of competent advice	ASE*, CLEAPSS*, MISAC, NCBE, SSERC* (*members only)

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Factor	Relevance
Useful check list	*CLEAPSS Laboratory Handbook, ASE Topics in Safety*, 2nd edition (ASE 1988), pp. 34–37
Essential reference	*ASE Topics in Safety*, 3rd edition (ASE 2001)

Key to abbreviations: ACDP Advisory Committee on Dangerous Pathogens, *ASE* Association for Science Education, *CLEAPSS* Consortium of Local Education Authorities for the Provision of Science Services, *MISAC* Microbiology in Schools Advisory Committee, *NCBE* National Centre for Biotechnology Education, *SSERC* Scottish Schools Equipment Research Centre

1.4 Spillage Management

1.4.1 Spills

The spillages cultures are immediately reported to the teacher/researchers or lab technicians for dealt with quickly. Spilled cultures and adjacent debris (glass, cotton wool plugs, etc.) **must not** be touched with unprotected hands/open-handed. **Must** wear disposable gloves and cover the disinfect spill area with several layers of cloth/towel drenched in a proper disinfectant and leave for at least 15–30 min. Immediately spill debris cleaned into a container/dustpan using towels. Transferring all disposable materials (e.g., roasting bag) into suitable container for autoclaving and disposal. A container must be sterilized or decontaminated by autoclaving or by soaking (at least 24 h) in hypochlorite (sodium chlorate I).

1.4.2 Broken Glass

The broken glass must be cleaned prudently into proper container, autoclaved and disposed of in a lesion-proof container.

1.4.3 Splashes on Clothing and the Skin

The contaminant clothes should be waterlogged in disinfectant. Splashes on the skin should be cured as quickly; hot water and soap may be sufficient for washing; if necessary the skin can be disinfected.

Hint
It is more useful to use spillage kit always ready at hand. The components are suggested in below:

- Beaker for making fresh disinfectant
- Disposable gloves
- Container/Dustpan and brush
- Towel/cloth
- Autoclave/roasting bag

1.5 Resources

1.5.1 Equipment (Source: ASE 2001)

Equipment	Use
Loops or needles	Routinely inoculate of agar slopes/deeps and lesser volumes of liquid media (up to *ca* 10 cm^3)
Straight wire	Inoculation from very small colonies; transfer of small inocula from liquid media
Spreader (glass/plastic)	Making lawn/spread plates
Forceps (metal/plastic)	Transfer of sterile paper/antibiotic discs; also plant material, e.g., short lengths of root with nodules
Pipette	Transfer appropriate volumes/drops of culture/sterile solutions
Teat	Filling and tipping pipettes safely (**never** pipette by mouth)
Test tube	Small volumes of liquid media/agar slants/sterile solutions for inoculation (held in test tube rack; dry nonabsorbent cotton wool plug or plastic cap prevents contamination)
Universal bottle (wide neck); McCartney bottle (narrow neck)	Volumes of liquid media and agar media/sterile solutions for inoculation or storing sterile media or stock cultures on agar slants (keep upright on bench; plastic screw cap thwarts contamination and reduces aerosol during long storage)
Conical flask	Medium or large volumes of liquid media for inoculation and liquid/media for short-term storage (nonabsorbent cotton wool plug prevents contamination but never reduce evaporation during long storage)
Petri dish (plastic/glass)	Plastic: Presterilized for streak/spread/lawn/pour plates; Glass: Only materials for sterilization by hot air oven, e.g., paper discs
Marker pen	Labeling petri dishes, test tubes, flasks, bottles, and microscopic slides

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Equipment	Use
Personal protective equipment (level 2, level 3, *Topics in Safety*, 3rd edition (ASE 2001)	Clean laboratory coat/apron: Protection of clothing, containment of dust on clothing; Safety spectacles: Not considered essential when dealing with suitable cultures and observing GMLP but may be required by local regulations and for dealing with chemicals.

1.5.2 Apparatus (Source: ASE 2001)

Apparatus	Use
Bunsen burner	Disinfection of needle/wire loops, metallic forceps and glass spreaders
Waterproof sheet or tray	Provides each student working area if the bench surface is not properly sealed
Autoclave/pressure cooker	Sterilization of all media, solutions, and equipment before use
Gas ring/hot plate	Steam generation in autoclave
Autoclavable/roasting bag	Holds contaminated items in autoclave to contain spillages
Hot air oven	Sterilization of glass petri dishes, pipettes, and paper discs
Discard containers containing disinfectant	Disposal of the used pipettes and slides of nonstained microscopically preparations
Microwave oven	Melting solidified agar media for use
Incubator	To incubate the cultures
Water bath	Meet the specific temperature for keeping agar media molten for use; exact temperature control
Thermometer	Checking incubator/water bath/laboratory temperatures
pH meter	Checking and adjusting pH values of media and solution
Cupboard	Storage of culture media and stock cultures
Refrigerator	Storage of heat-labile materials
Microscope, slides, cover slips, stains, staining rack, immersion oil	For microscopically examination

1.5.3 Materials (Source: ASE 2001)

Materials	Use
Culture media ingredients	Stock wide range of culture media in dehydrated form (powder/tablets)
Disinfectants	Decontaminant the surface of workbenches before and after use and spillages; always disposal of the used pipettes and slides. In soap use for hand washing
Ethanol (70% industrial methylated spirit)	Sterilization of metal forceps and glass spreaders by explosion
Autoclave indicator tape	Changes color in response to heat to distinguish those items that have received heat treatment (although it is not effective sterilization)
Steriliser control tube/strip	Changes color when accurate temperature applied and held for the obligatory length of time to effect sterilization
Non absorbent cotton wool	Plugs for test tubes, flasks, and pipettes
Spillage kit	Dealing with spilled cultures

1.6 Flaming the Needle/Loop

1. For sterilization of needle/loop, first hold the needle/loop in the flame of the Bunsen burner to kill all infecting substances/organisms.
2. The needle/loop can be made red-hot for a few seconds (Fig. 1.1).
3. After flaming, make sure that the needle/loop is slightly cool before taking sample from the inoculum culture (for transferring culture).

Fig. 1.1 Demonstration of the flaming the needle/loop

4. When transferring the fungal colony from cultural plate, need to cool the needle/loop by touching on the edge of agar. Again when fungal colony transferred from broth media, the red-hot loop/needle can create a sizzling noise.
5. Subsequently the needle/loop can spontaneously be cooled once it touch with the broth culture, wait for 1 or 2 s before take away the needle/loop of inoculum from the bottle/tube (avoid dipping hot needle/loop into media because this always creates aerosols).

1.7 Flaming the Neck of Bottles/Test Tubes

The neck of bottle/tube is passed through the flame of Bunsen burner to create a convection current which forces air out of the bottle/tube. Avoid the airborne contaminations from the bottle/tube. Heat of the Bunsen burner causes the air near by work area to increase, reducing the accidental airborne microbes contaminating cultures.

1. Loosen the cover/cap of the bottles/tubes so it can be removed easily (Fig. 1.2).
2. Hold the bottle/test tube with left hand.
3. Take out the cover/cap of the bottle/cotton wool plug with little finger of the right hand.
4. Don't put down the cover/cap or cotton wool plug.
5. Flame the bottle/test tube neck (forward and back) through a hot Bunsen flame.
6. The mandatory procedures are always followed, e.g., removing culture, replace the cover/cap on the bottle/cotton wool plug using the little finger (turn the bottle, not the cap).

Hints
- Label the tubes/bottles in a place that cannot clean off in handling. Use marker pens or self-adhesive labels.
- Cotton wool plugs may accidentally catch fire. If so, immediate cover with lid or dry cloth, avoid blowing or soaking in water.

1.8 Media

1.8.1 Preparation of Culture Media

The prepareation of agar media is followed according to manufacturer's instructions. Before sterilization, make sure all elements are entirely melted by using microwave oven heat if further required. Avoid wastage by preparing only sufficient for either immediately use or use in the near future. Usually authorize about

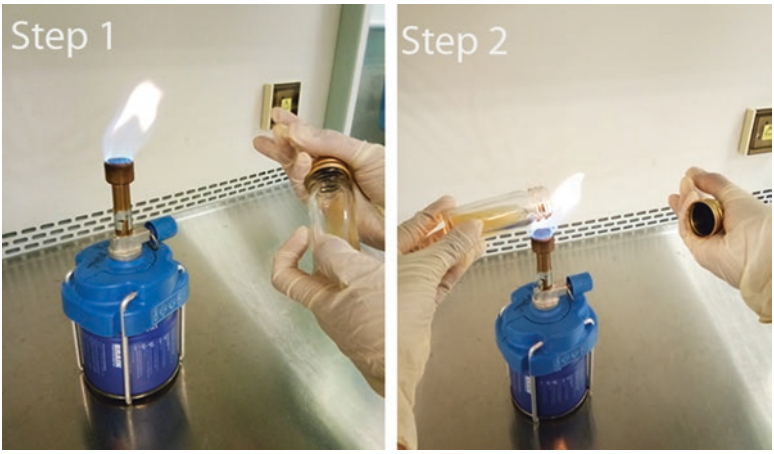

Fig. 1.2 Flaming the neck of bottles/test tubes

15–20 cm^3 medium/petri dish. Agar slopes are prepared with universal bottles allowing sterile molten media to solidify in a sloped position.

1.8.2 Pouring a Petri Plate

1. Sterile molten agar bottle is used from the incubator at 45 °C.
2. Hold the bottle in the left hand and remove the lid using the right hand little finger.
3. Flame the bottle neck by passing through back and forward of neck.
4. Remove the lid/cover of the petri dish using the right hand and pour around 15 mL of the sterile molten agar into petri dish and replace the lid/cover of the petri dish (Fig. 1.3).
5. After that, flame the bottle neck and cover with the lid.
6. Smoothly rotate the petri dish to make sure that the whole media is equally dispersed in the plate.
7. Keep the petri dish for few minutes for solidification.
8. Properly seal the petri dish using Parafilm, and incubate in an inverted position.

1.8.3 Agar Slants

The growth of fungal colonies are transferred into agar slants, in addition, uncertainty found any excess broth in the bottle/tube and agar plates, it should be removed. Univerial bottle/tube containing agar should be solid formed that call a agar slant. A needle/loop can be used to inoculate an agar slant by stabbing the needle containing inoculum into the agar (Fig. 1.4).

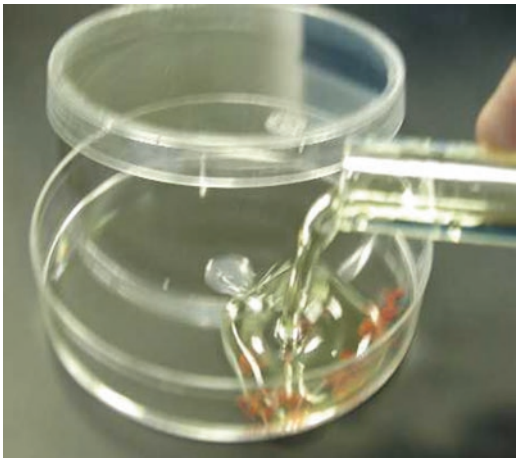

Fig. 1.3 Pouring PDA onto petri plate

Fig. 1.4 Example of agar slants

1.8.4 Storage of Prepared Media

Blue screw-capped bottles of agar and broth should be kept at temperatures of 12–l6 °C for 6 months. When reuse the kept agar media, it should be remelt again using hot water or streaming bath or microwave oven or pressure cooker. After completely melted, the molten agar can be kept in incubator at least 50 °C for ready to use or further uses. All stored media should be kept away from light. After pouring agar in the plates, the plate should be sealed with parafilm and put in well-sealed plastic bags, then kept at 3–8 °C for further reuse.

Freshly prepared media are always better than stored media. It is very important to avoid prolong storage times. Labile beta-lactam selective agents have very short active lives and the media containing some elements that needed within a few days of preparation. A good laboratory practice always supports the shelf-lives of all media preparations and also postmarks the containers or boxes accordingly. Otherwise poor fungal growth results from loss of moisture from agar plates.

Make sure that all agar plates are incubated in a moist environment. Look through the contamination symptom, color changes, irregular filling or bubbles on surface of agar, hemolysis, and symbols of dryness such as cracking, shrinking, and loss of volume. Any defective plates or tubes should be removed.

1.9 Disinfectants

Specific disinfectants are used for the specific purposes of working strengths. Disinfection is defined as the destruction, inhibition, or removal of microorganisms that may cause disease or other difficulties, e.g., spoilage. The use of chemicals also disinfectants occurred.

The available disinfectants and their uses are listed here (Source: ASE 2001).

Disinfectant	Use	Working strength
VirKon	Worktable, discard pots for pipettes and slides, skin disinfection Spillages	1% (v/v) Powder
Hypochlorite (sodium chlorate I)	Discard pots for pipettes and slides	2500 ppm (0.25%, v/v) available chlorine
Ethanol	Skin disinfection	70% (v/v) industrial methylated spirit

- When making the strength of working solutions from stock with dealing powder form, wear eye protection and gloves to avoid irritant or harmful effects.
- The uses of disinfectants at working strength newly made from powder form or stock strength.
- The activity of VirKon solution may obtain until 1 week or less, e.g., 1 day, after use. The working strength of hypochlorite is prepared on the day.

1.10 Incubation

1. The lid and agar plate can be taped together with 2–4 short strips of Parafilm as protection from accidently (or unauthorized) opening during incubation.
2. Agar plates should be seeded with uppermost if not the molten agar can be dripped on the lid and the culture. Fungal colonies may cause and spread to each other and the risk of spillage of the contaminated liquid.

3. Incubators are set up at the specific temperatures and avoid the cultures being interfered with or accidentally discarded. Many fungal cultures grow optimally at room temperature 28 ± 2 °C.

1.11 Accidents

1. Researchers/scientists/technicians must evacuate from the contamined work area, e.g., breakage of culture tubes, etc., the workplace area should be completely decontaminated.
2. You should follow several steps if a culture tube/plate is broken.
   (a) All staffs should immediately evacuate the workplace..
   (b) Proper PPE must be worn from the decontaminated room.
   (c) The room and cover spill is removed using paper towels properly soaking with 2% Amphyl solution (as disinfectant). Before cleaning up the spill, the towels should be left for at least 1 h; after that keep the wet area with Amphyl to protect dried particles becoming airborne.
   (d) Leave from worktable area and do not reenter the laboratory or workplace for at least 1 h.
   (e) Contaminated clothing should be autoclaved for 1 h at 121 °C.
3. The laboratory technician or administrator must be informed if any accident happens and at the same time complete the incident report.
4. If accidents happen to the eye,
   (a) Go immediately to the eye wash station and call for assistants.
   (b) Thoroughly wash eyes for at least 20 min to remove all chemical particles.
   (c) Look for medical assistance.

1.12 Emergency Preparation and Response

Emergencies such as explores, hurricanes, and other disasters, its happen normal working environment. The realistic disruptions are commonly occurred as exposures, injuries, spills, equipment failure, electricity power, fire, water loss, or flooding. Handling each of these emergencies and disturptions can follow each institutional guidelines. However, keep a written emergency and evacuation guilde line in workplace and talk to all personnel for such circumstances to avoid worker injury or contamination via infectious agents.

The microbiology laboratory should take the following steps for emergency:

1. All doors should be made of wood.
2. All hitech equipment, refrigerators, and freezers should be provided with emergency electrical outlets or red sockets.

3. Incubators should be locked to prevent breakage.
4. Disconnected computer and electronic equipment are covered with plastic and transferred to one room.
5. Log books and other paperworks should also be covered with plastic andkept in one room.

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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

S. Siddiquee, *Practical Handbook of the Biology and Molecular Diversity of Trichoderma Species from Tropical Regions*, Fungal Biology,
DOI 10.1007/978-3-319-64946-7_2

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, Pencillium* 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 dexon does not inhibit the growth of *Trichoderma* colonies on the media. In the presence of Dexon 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 $MgSO_4 \bullet 7H_2O$
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 Erlehnmeyer 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 quintozene 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 $MgSO_4 \bullet 7H2O$
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 quintozene, 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 $MgSO_4 \bullet 7H_2O$
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/m^2 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.

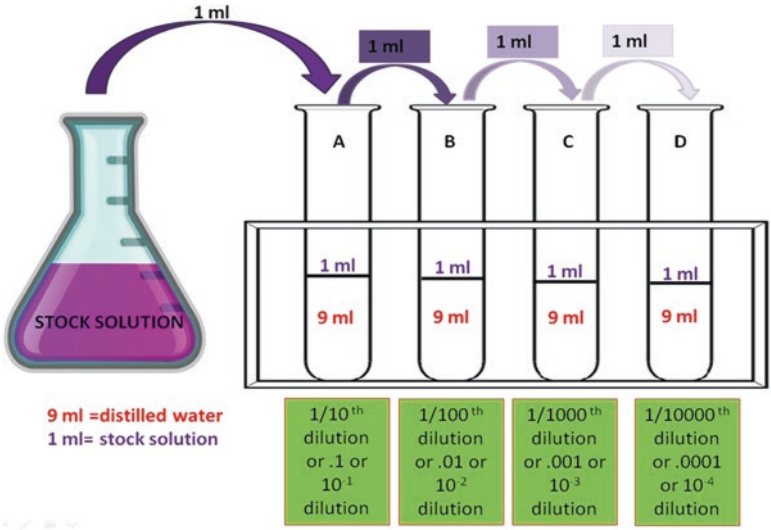

Fig. 2.2 Procedures of Serial dilution in microbiology

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).

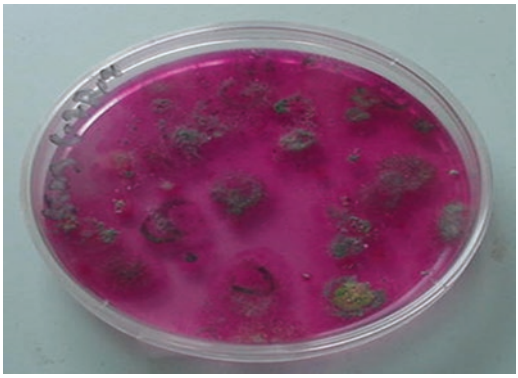

Fig. 2.3 Seven-day old culture showing *Trichoderma* CFU on TSM

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 Erlehnmeyer 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/m^2 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).

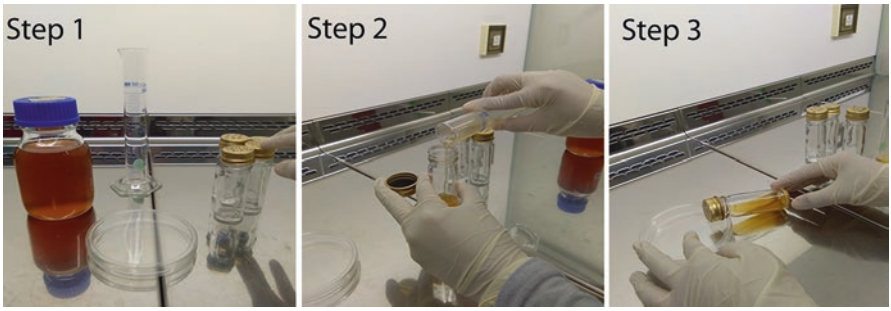

Fig. 2.4 Step by step preparation of slant

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 1×106 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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Chapter 3
Slide Culturing of *Trichoderma* Isolates

3.1 Introduction

The isolation, culture, and microscopic observation of *Trichoderma* species require the use of more suitable media and slide culture techniques. Slide culture remains the mainstay of morphology identification of *Trichoderma* species. The various stratums are observed by the naked eye through the light and compound microscopes. Using compound microscope, the identification of *Trichoderma* isolates may be possible the given name until the genus due to examine their diverse morphological characters in agar cultures.

The process of transferring mycelia into a slide normally breaks up the structure of conidia, conidiophore, chlamydospore and phialides, for that reason, *Trichoderma* isolates become very difficult at the species level identification. However, slide cultures preserve the morphological characteristics relatively undisturbed compared to other techniques such as cello-tape mount and tease mount method. Apart from, it is a simple and rapid method for the characterization and identification of *Trichoderma* isolates. Isolates are commonly identified by close examination of their morphological and physiological characteristics. Thus, isolates are directly grown on a thin film of agar (Cornmeal Agar (CA), Potato Dextrose Agar (PDA), and Sabouraud Agar (SA)) on the glass slide. There is a need small portion of spores/mycelia from the culture agar plate then transfer to the slide. Thus, the morphological observations are the preliminary step for the identification of *Trichoderma* isolates at the genus level so it cannot be neglected.

S. Siddiquee, *Practical Handbook of the Biology and Molecular Diversity of Trichoderma Species from Tropical Regions*, Fungal Biology,
DOI 10.1007/978-3-319-64946-7_3

3.2 Materials

Materials that are needed for the preparation of slide culture:

1. Sterile petri dish.
2. Microscopic slides and coverslips (Sterile).
3. Thin layer of CA or PDA or SA (6–10 mL).
4. U-shaped glass rod.
5. Scalpel.
6. Inoculating needle.
7. Forceps.
8. Bunsen burner.
9. Sterile distilled water.
10. 95% alcohol.
11. Lactophenol cotton blue.
12. Nail polish.
13. Filter papers (9 cm diameter of Whatman grade 1 filter papers).

3.3 Procedure

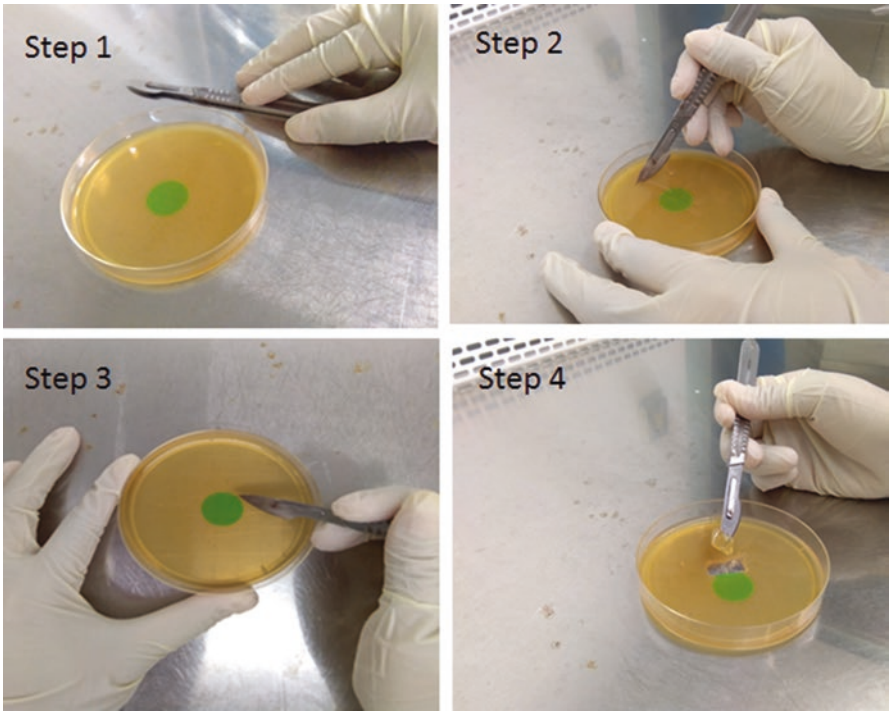
There are two different methods of slide culture that can be used for the microscopic observation of *Trichoderma* isolates. All of the processes are conducted in the Biosafety Level 2 (BSL-2).

3.3.1 Slide Culture Technique I

1. Aseptically keep a sheet of sterile filter paper in the bottom of sterile petri dish (Glass petri dish) using sterile forceps.
2. A sterile U-shaped glass rod is placed on the top of the Whatmann filter paper to form a platform for the slide.
3. Pour a proper amount of sterile distilled water (approximately 3 mL) on the filter paper to entirely soak it.
4. A sterile glass slide is placed on the U-shaped glass rod by sterile forceps.
5. Use a sterile scalpel blade to excise a 1 cm square agar block from the thin layer (6–10 mm thick) of suitable media.
6. Pick up the square block using the flat side of scalpel, then carefully transfer the block aseptically to the center of sterile glass slide.
7. Firstly, flame the inoculating loop, then allow it to cool before inserting it into slant culture (*Trichoderma* isolates). When obtaining the spores or mycelial fragments by loop after allow it touch four sides of agar block for examination. Do not take the spores or mycelial from the center of *Trichoderma* colony because of over-maturity.

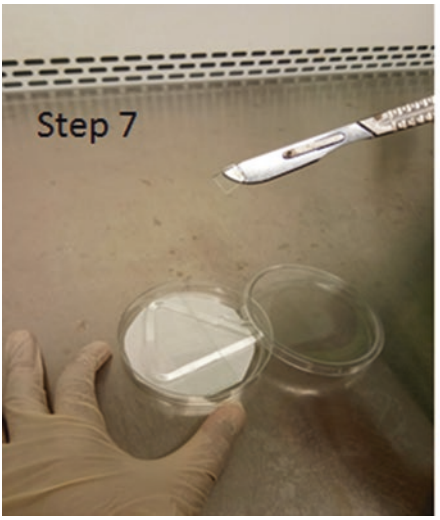
8. Sterilize the cover slips by submerging with alcohol. Using a sterile forceps, take out and flame the coverslips to burn off the alcohol then cool and place it on the top of the agar block.
9. Incubate the slide culture at room temperature condition of 28 ± 2 °C.
10. After 48 h, the slide culture should be examined at lower magnification. If the growth of slide culture is an inadequate and spores are not clear appeared, allow them to grow for another 24–48 h before examining.
11. When sufficient amount of growth has occurred, then add a few drops of sterile distilled water to the center of the new clean microscope slide (Lactophenol Cotton Blue can be used instead of sterile distilled water if available).
12. Remove the coverslip and place it, with the growth uppermost onto the drop of distilled water in the glass slide.
13. Observe under low and high magnifications of light and compound microscopes.
14. Observations can focus on the size, shape and colour of the conidia, the branching pattern of the conidiosphores, phialides and the presence or absence of chlamydospores.

The following demonstrates slide culture technique I to observe undisturbed microscopic characteristics of *Trichoderma* isolates:



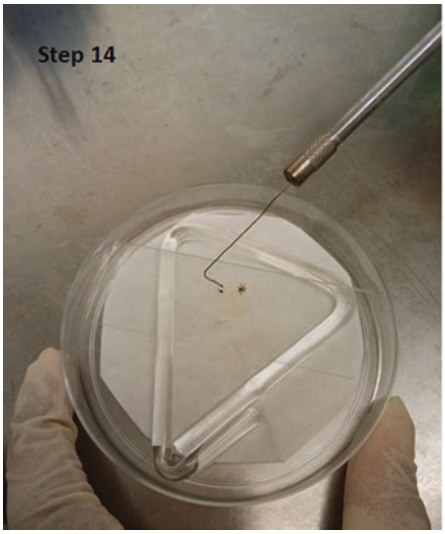
Censor the square agar block that is sufficient to fit on the glass slide.

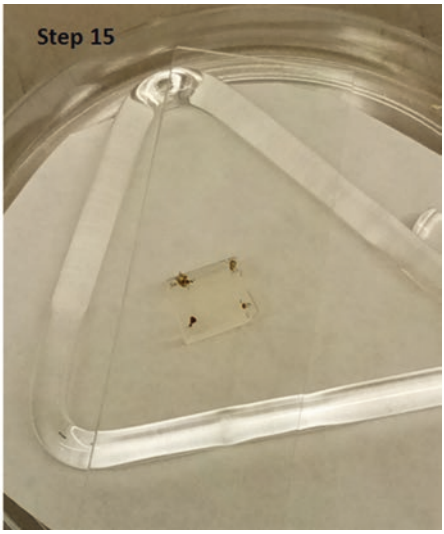
Use the scalpel to the agar block.



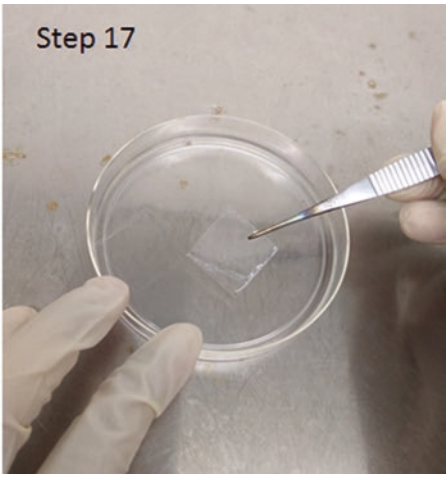
Carefully transfer the square agar block to the center of the glass slide.

Flame the loop, then allow it to cool before inserting it take out a small amount of spores/mycelia.

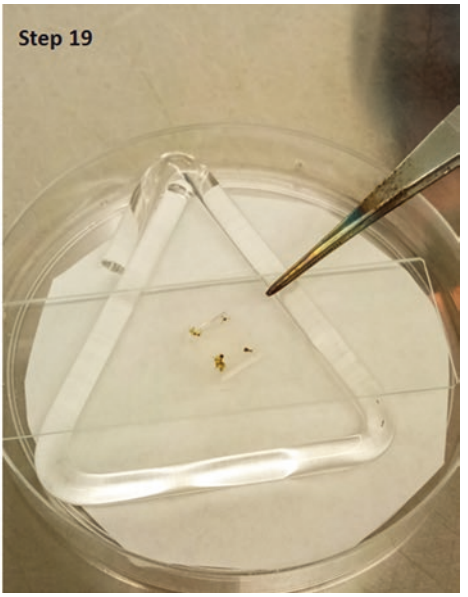




The growth of *Trichoderma* isolates are inoculated edge of the agar block (steps 14 and 15).

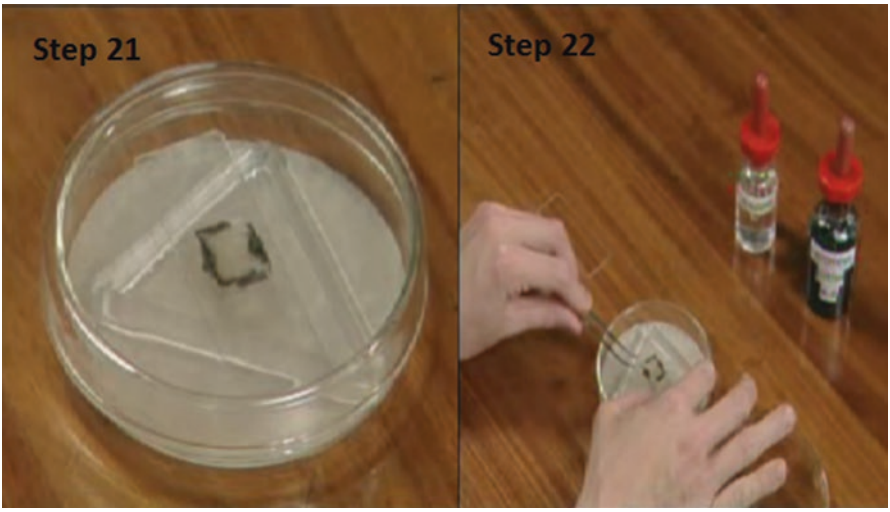


The coverslips are sterilized by submerging in 70% alcohol and picked up with sterile forceps.

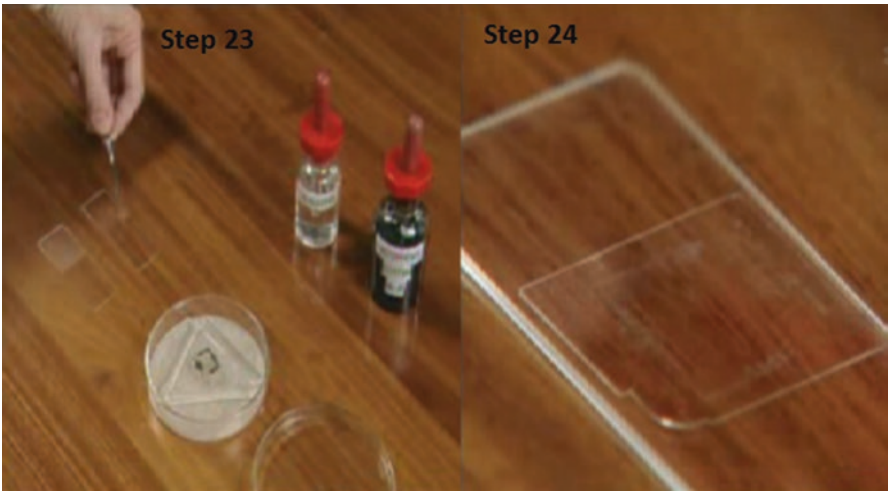


Shortly the coverslips are flamed to burn off the alcohol and placed on the top of the slide culture agar.

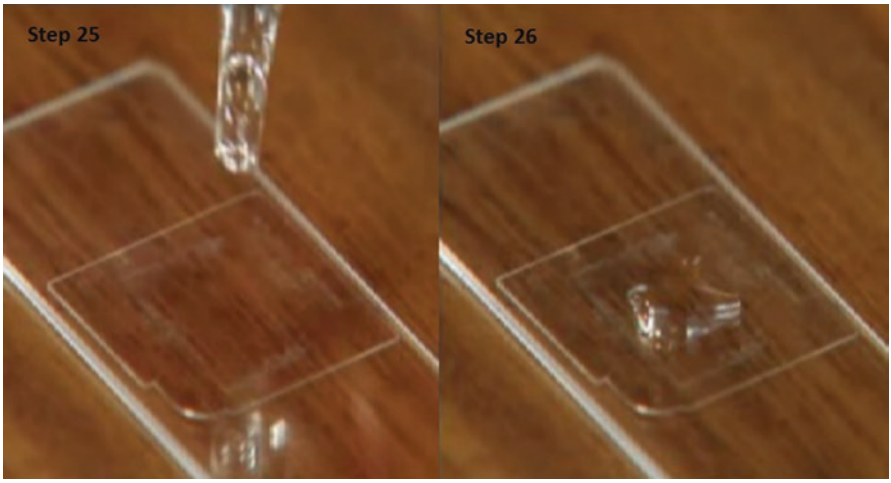
An approximately 3 mL of sterile distilled water should be added on the filter paper of the petri dish for keeping moisture to avoid the dryness of slide culture agar when incubating at room temperature.



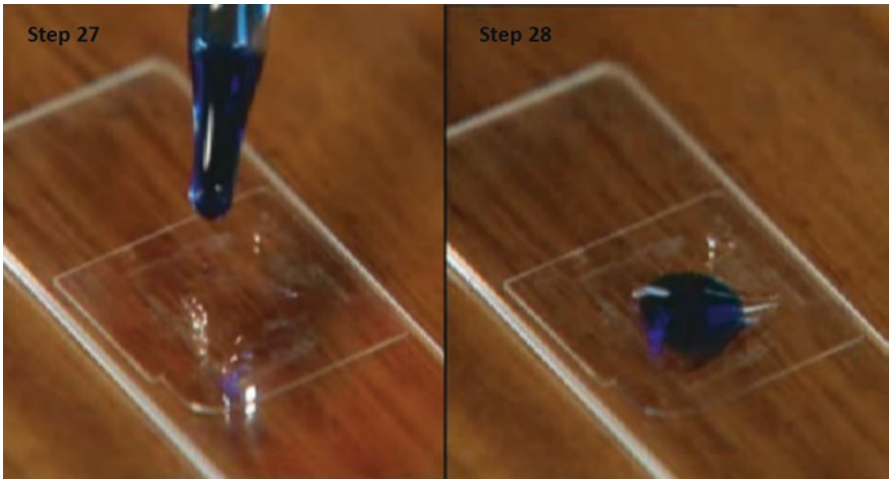
When sufficient growth has occurred, gently take away the coverslips.



The coverslips are placed on a new clean microscope slide with fungal growth uppermost.



Place a drop of sterile distilled water on the top of the coverslip.
Or



Add a drop of Lactophenol cotton blue on the coverslip (optional).

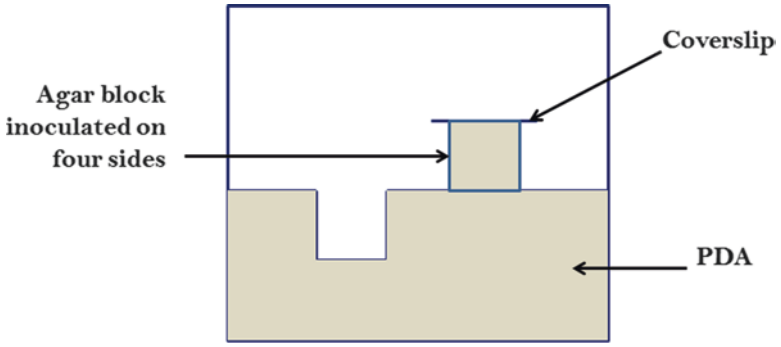
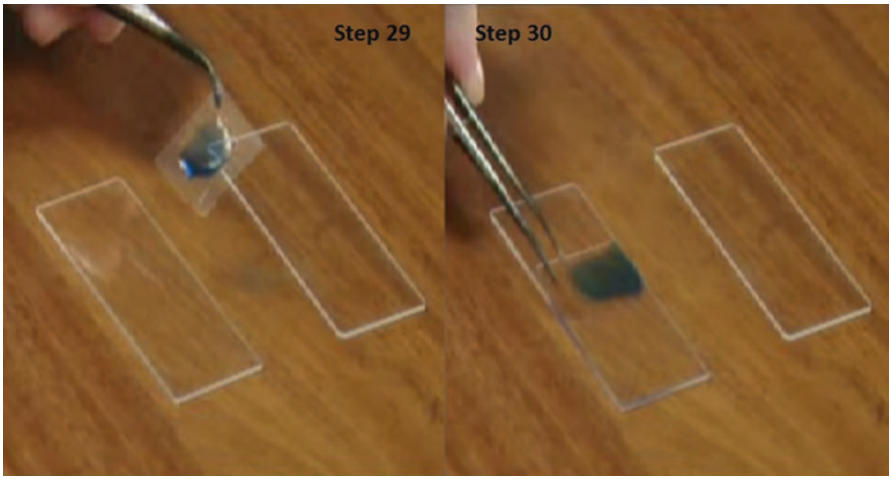


Fig. 3.1 Example of slide culture technique II to observe undisturbed microscopic characteristics of *Trichoderma* isolates



Turn over the coverslip and keep it onto a new sterile microscope slide. Observe under low and high magnifications.

3.3.2 Slide Culture Technique II

The correct identification of *Trichoderma* fungi is necessary to examine the proper arrangement of the conidiophores, conidia, phialides, and the presence or absence of chlamydospores. Slide culture technique II is described below with a simple modification using a single agar plate.

Slide Culture Technique II is usually a slight modification of the Slide Culture Technique I. Nutrient Agar or Potato Dextrose Agar is recommended for use in this method (Fig. 3.1).

1. Using a sterile blade, cut out the square agar block (2 × 2 mm) and place onto a sterile coverslip.
2. Flip the agar block up onto the surface of agar plate.
3. Inoculate the four sides of the agar block with spores or mycelial fragments of the isolates to be grown.
4. The coverslip is placed at the center of the agar block.
5. The plates are incubated at 28 °C until sufficient growth occurs.
6. Take away the coverslip from the agar block.
7. Add a drop of 95% alcohol as moistening agent.
8. Lower the coverslip onto a small sterile distilled water clean glass slide (or a drop of Lactophenol Cotton Blue).
9. The slide is left overnight to dry and then covered with fingernail polish.
10. Observe the culture slide under low and high magnifications.
11. Observations will focus on the size, shape, and color of the conidia, the arrangement of branching pattern of the conidiosphores, phialides, and the presence or absence of chlamydospores.

Chapter 4
Morphology-Based Characterization of *Trichoderma* Species

4.1 Introduction

The fungal kingdom is diverse, and it is estimated about 1.5 million species globally but only around 70,000 known species have been identified to date (Siddiquee 2014). Most of these species are classified as filamentous fungi. Several types of living organisms have been found from different substances that are utilized by humans for various applications. From these, an approximately 10% has been discovered and described until now. As morphology-based identification of fungi is still preliminary required for the given putative genus name. Hibbett et al. (2011) mentioned that still traditional morphology-based classification is authorized in the diverse classification of Fungi. The International Code of Nomenclature for algae, fungi, and plants (Melbourne Code) requires morphological Latin or English diagnosis for valid new fungal taxon descriptions (Norvell 2011). Microscopic observations are still a simple, economic, and efficient way to assay of morphological characters, and classification. The observations of the beauty of fungi are still enjoyable for many mycologists; they are very much excited to identify the anonymous species under microscope. In the past 10 years, new fungal species are identified averages of 1196 per year (Hibbett et al. 2011). Among these new fungal species are identified only morphology characters without prior DNA sequencing or molecular data from 1999 to 2009 (Hibbett et al. 2011). Combinations of physiological characteristics and microscopic observations still have practical and scientifical value in examining of fungi. Microscopic observations are commonly applied in fungal research and commercial laboratories.

Fungal species which belongs to the genus of *Trichoderma* (telemorph *Hypocrea*, Hypocreales, Ascomycota) was first introduced by Persoon in 1794. The genus of *Trichoderma* is found in soil, decaying wood, agricultural lands, prairie, forest, salt marsh, desert soils, manure and other form of plant organic matter throughout all climatic zones (Gams and Bissett 1998; Hoyos-Carvajal and Bissett 2011).

S. Siddiquee, *Practical Handbook of the Biology and Molecular Diversity of Trichoderma Species from Tropical Regions*, Fungal Biology,
DOI 10.1007/978-3-319-64946-7_4

The genus *Trichoderma* is easily grown with different media such as Potato Dextrose Agar (PDA), Blakeslee's Agar (BLA), Malt Extract Agar (MEA), Cornmeal Dextrose Agar (CMD), and Czapek Dox Agar (CDA) (Bissett 1991). The colony appearances of *Trichoderma* species are diverse according to the type of media used. According to Samuels et al. (2002), there are slight differences on the colony appearance of *Trichoderma* when they are grown onto PDA and also in CMD. They reported that *Trichoderma* species are white on rich media such as PDA and more transparent when grown on media such as CMD (Samuels et al. 2002; Shah et al. 2012). And they also found that the mycelia development and pigmentation can be better observed on PDA compared to other media (Samuels et al. 2002). Scattered blue-green or yellow-green pigment on the colony becomes observable when conidia are formed. Occasionally, they observed concentric rings made by these pigmentations by some *Trichoderma* species (Samuels et al. 2002). For example, *T. harzianum* formed 1–2 concentric rings with green conidial production when grown on PDA but no concentric ring has observed by *T. viride* and *T. pseudokoningii* (Shah et al. 2012). The green conidia are generally a diagnostic of the genus but green conidia also have been found in the unrelated genera such as *Penicillium* and *Aspergillus* (Jaklitsch et al. 2006). Some species are characterized by a complete lack of pigment in reverse, where reddish brown pigment occurs in reverse for some species (Gams and Bissett 1998). The colonies of some *Trichoderma* species on PDA are shown in Fig. 4.1. Furthermore, indistinct moldy or musty odors are commonly produced by different *Trichoderma* strains. Some species of *Trichoderma* such as *T. viride* is produced a sweet smell resembling "coconut" odor (Gams and Bissett 1998, 2002; Samuels et al. 2002).

Majority of *Trichoderma* species grown well at 25–35 °C, but some species grow well above 35 °C also (Samuels et al. 2002). Growth rates in culture can be useful to distinguish between morphologically similar species (Gams and Bissett 1998). For example, *T. harzianum* can be distinguished from the morphologically similar species of *T. aggressivum* and *T. atroviride* by growing at 35 °C (Samuels et al. 2002). After 4 days of incubation, neither *T. aggressivum* nor *T. atroviride* colony grew more than 5 mm while *T. harzianum* grew well and sporulated at 35 °C (Samuels 2004).

Trichoderma species are very diverse in their morphological characters. It produces numerous spores (conidia) which are mostly green depending on the cultural agar (Howell 2003). The backside of the colonies are often colorless, buff, yellow, amber of yellow-green and also many species produced thick-walled spores (chlamydospores) in immersed mycelium (Gams and Bissett 1998). They are decomposers of woody material due to its classification as the imperfect fungi (unknown sexual stage), fast-growing fungi with rapid growth rate, dominant in soil with a wide range of substrates, and are an aggressive competitor in nature (Howell 2003).

Microscopic observations such as spores and their arrangement, conidiophores, conidia, phialide, and chlamydospore are very important in the classification of species of *Trichoderma* isolates. The colonies of *Trichoderma* have the key characters such as growth rate, growth pattern, pigmentation, pustule formation, and odors that can be used to identify as *Trichoderma* species.

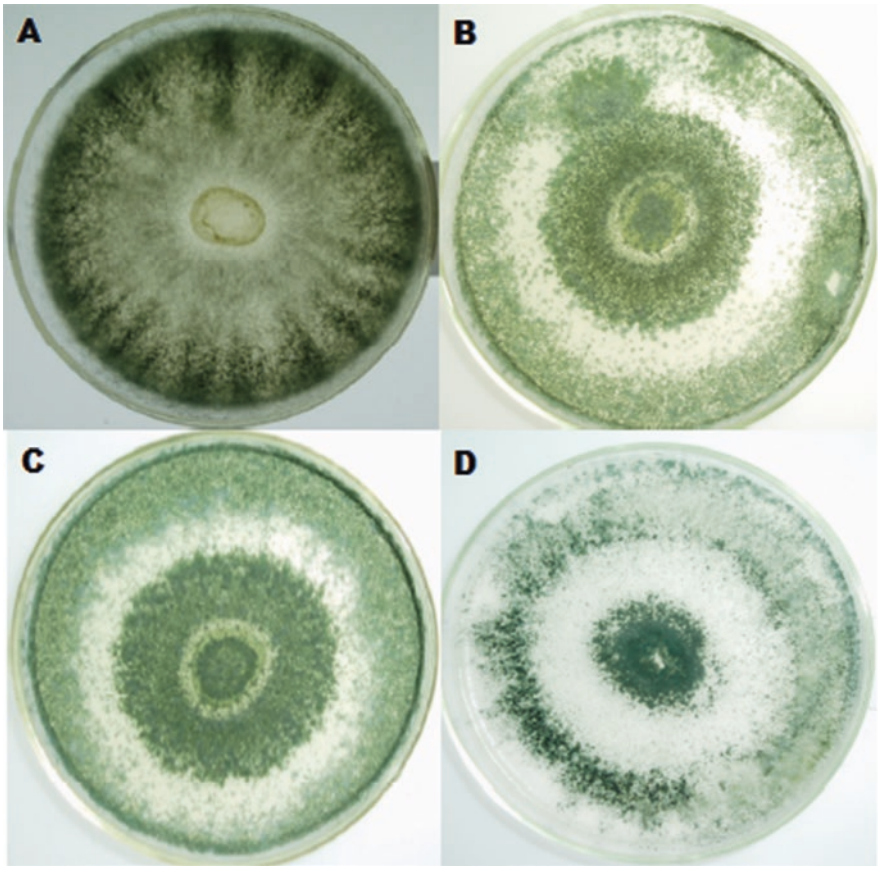


Fig. 4.1 Colony appearance of *Trichoderma* species are grown on PDA for 7 days where picture (**a**) is *T. atroviride*, (**b**) is *T. longibrachiatum*, (**c**) is *T. virens*, and (**d**) is *T. harzianum* (Source: Zhang and Wang 2012)

4.2 Definitions and Taxonomic Value of Morphological Traits

The following phenotype descriptions are mandatory to know the terminology definitions as *Trichoderma* mycologists; all terminology definitions descriptions are included in this book even through previously defined by Jaklitsch (2009):

4.2.1 Teleomorph

Asci: are not diagnostic traits, their size is mostly dependent on ascospore size; the length of the stipe is measured. Asci can see much wider in thin KOH mounts shortly before drying out.

Ascospores: tiny in size, however, some individual species can be defined using this character. Green ascospores turn brown in KOH, and appear often too brown in photographs taken from mounts in KOH; the color in KOH is also dependent on the thickness of mounts, i.e., in thinner mounts ascospores appear more distinctly brown.

Ostiolar dots/regions/areas: are measured in dry stromata. They include the ostiolar opening and a part of the surrounding cortex of the stroma, which appears darkened using a hand lens. Sometimes perithecial contours are parts of ostiolar dots. Ostiolar dots are commonly inconspicuous in teleomorphs of *Trichoderma* sect. *Trichoderma*. True ostioles, i.e., the ostiolar openings, are colorless, generally minute, inconspicuous or invisible. The apical width of ostioles in section refers more correctly to the ostiolar width at the stroma surface; ostioles may be attenuated at the true apex; the width includes the wall of the ostioles, except where noted. Ostiolar dots are of limited value in species delimitation, although typical, fresh material of certain species can often easily be identified using this character: e.g., fine, plane dots densely set in large numbers as in *H. strictipilosa* vs. *large*, projecting dots in small numbers as in *H. sinuosa*.

Perithecia: are immersed in a single layer in the upper part of the stroma. The perithecial height measured in median section includes the apex located at 1/3–1/2(−2/3) of the lower part of the ostiolar canal. Perithecia appear larger in crush mounts than in sections. There is considerable variation in the size of perithecia, but ranges are overlapping. The perithecial wall may be colorless or pigmented or change its color in KOH (e.g., *H. thelephoricola* vs. *H. sinuosa*).

Stroma: is a more or less pseudoparenchymatous fungal tissue containing usually more than one perithecium; highly conserved in *Hypocrea*, i.e., not particularly informative in many species. Stroma is used in a broad sense, i.e., including also entirely prosenchymatous ("hyphal") stromata, more correctly described as subicula that form usually widely effused mats.

Stromata, fresh/dry, immature/mature: Stromata in nature may be fresh, dry, or rewetted. They may be immature, mature or overmature. Their appearance is often strongly dependent on climate conditions. Fresh stromata are soft. Their sizes only estimates in the field. They often shrink considerably upon drying. The mature state of stromata may be indicated by green, white or yellow, floccose, granular or filiform ascospore deposits. These should not be mistaken for white or green floccules caused by conidiophores of the anamorph often present on immature, sometimes also (over-)mature stromata.

Stromata, colors: are variable and difficult to define, but are useful for preliminary identification of species. Color changes upon drying of fresh stromata can be diagnostic. Drying in nature poses difficulties, e.g., originally yellow stromata may be already reddish brown at the time of collection. In addition, the presence of only one developmental state of stromata in a specimen makes even preliminary identification difficult. Usually some uncertainty remains, particularly in species with yellow, pulvinate stromata, or reddish brown to brown, pulvinate stromata.

Stromatic tissues: are studied in vertical section. The morphology of the several parts of the stroma may be diagnostic, particularly the cortical tissue, e.g., pseudoparenchymatous in *H. aeruginea*, prosenchymatous in *H. danica* and *H. spinulosa*.

4.2.2 Cultures

Autolytic activity: is a semiquantitative estimation of usually circular colorless excretions at tips or less commonly at septa of mainly superficially growing hyphae; often they are accompanied by death of hyphal segments. In some species, autolytic excretions and dying hyphae become yellow, reddish or brown.

Coilings: are circular-oriented parts of aerial hyphae or vegetative hyphae on the agar surface. These structures are absent to abundant, commonly more abundant on PDA (sometimes on Spezieller nahrstoffarmer agar, SNA) than on other media.

Color of colony reverse: is determined on white background. Usually no or little discoloration of CMD (cornmeal agar plates supplemented with 2% (w/v) D(+)-glucose-monohydrate (CMD)), and SNA (synthetic nutrient-poor agar), are caused by growth of *Hypocrea*/*Trichoderma*, with few exceptions, e.g., green color by *H. aeruginea*. Pigments are more frequently formed on PDA; typically yellow, brown, less commonly reddish colors are distinguished. However, formation may vary among isolates and may be absent in old cultures.

4.2.3 Growth Media

The compatibility with studies is selected by Samuels et al. (2006) in below:

CMD: has provided good growth, long-term vitality, and good conidiation, but conidiation often degenerates and may be absent after several subsequent transfers.

PDA: has provided good growth, usually abundant mycelium, but conidiation is frequently effuse and ill defined, often remaining colorless or white in green-conidial species. However, this medium often provides diagnostic macroscopic growth/conidiation patterns.

SNA: often comparable with CMD, it yields cultures of low biomass, with hyphae degenerating soon. However, the main advantage of SNA over CMD is a more reliable and transfer resistant conidiation. Conidiation morphology on SNA and CMD is usually in accordance with that found in nature.

4.2.4 Growth Plates/Colonies

Growth plates/colonies: centre of the colony = area around the inoculation plug (called plug in descriptions); proximal area = area behind the plug, i.e., short growth distance to the margin of the petri dish; distal areas = opposite proximal areas, i.e., long growth distance to the margin of the petri dish; middle of the colony = centre of the plate. Lateral areas = areas close to the lateral margins of the petri dish. Margin stated without further specification denotes the distal colony margin.

Hyphae, primary: are the first hyphae leaving the inoculation plug, beginning the principal hyphal network. These are often conspicuously wider than secondary hyphae.

Hyphae, secondary: are branches originating from primary hyphae; these are usually narrower than or of similar size as primary hyphae. Relative differences in hyphal width are characteristic for many species. The value of this trait is limited by the large number of *Hypocrea*/*Trichoderma* species.

Odor of colonies: is of limited use for identification. Coconut like odor on CMD and PDA, caused by the antifungal antibiotic 6-pentyl-α-pyrone, is typical for some species of sect. *Trichoderma*. Other odors may be formed on PDA, but experience is required for their detection, because odors are difficult to quantify and most people are neither able to perceive specific odors nor link them to known odors.

4.2.5 Growth Plates/Conidiation

The following scenarios are found in *Trichoderma*:

1. Pustulate conidiation only, i.e., complex branching in tufts, pustules, shrubs, or granules
2. Pustulate conidiation preceded by effuse conidiation
   (a) Effuse conidiation on conidiophores with less complex branching than in pustules, otherwise similar to pustulate conidiation, with phialides and conidia being in the same range or phialides tending to be slightly longer and narrower than in pustules.
   (b) Effuse conidiation on simple, mostly verticillium-like, conidiophores different from pustulate conidiation in shape, size and arrangement of phialides, terminal branches and/or conidia (“synanamorph”) (Chaverri and Samuels 2003)
3. Effuse conidiation only, often starting shortly after the onset of growth (typical for *T.* sect. *Hypocreanum*).

4.2.6 Conidial Heads, Wet Versus Dry

Conidial heads, wet vs. dry: wet heads occur in many species, often they can be only seen on plates with the lid attached by parafilm, and removal of the lid may result in immediate drying of heads.

4.2.7 Conidiophores/Trees, Regular

Conidiophores/trees, regular: means a tree-like or more or less pyramidal shape (such as *Picea abies*), attenuated upwards, branches mostly paired and increasing in length from the top down, branches substituted by phialides at and near the top. The term trees is mostly used for side branches or ends of conidiophores having this shape as is common in *Trichoderma*. In pustules, conidiophores or trees are referred to the terminal branching system on the periphery of the pustule.

4.2.8 Conidiophore Terminology (Source: Jaklitsch 2009)

The following standard characters were noted and measured: type of anamorph (*Acremonium*- see Fig. 4.2, *verticillium-, gliocladium-, pachybasium-, or trichoderma* like; see Fig. 4.3) previously described by Jaklitsch (2009)

1. ***Acremonium-like*:** is the least complex, short, effuse type of conidiation in *Trichoderma*. The conidiophores consist of a stipe of one to a few cells, with one or few phialides originating directly from this stipe on a single level. This conidiation structure is typical for sect. *Hypocreanum*, where conidiation is initially acremonium-like but usually develops into a verticillium-like structure bearing long phialides.

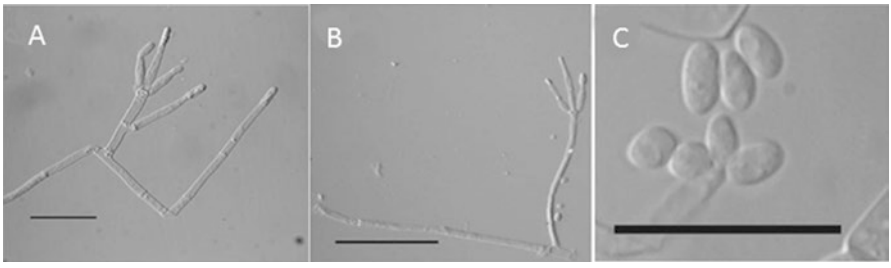


Fig. 4.2 Selected examples are showed the conidiosphore (**a**, **b**) and conidia (**c**) of *Trichoderma* species which is *Acremonium*-like

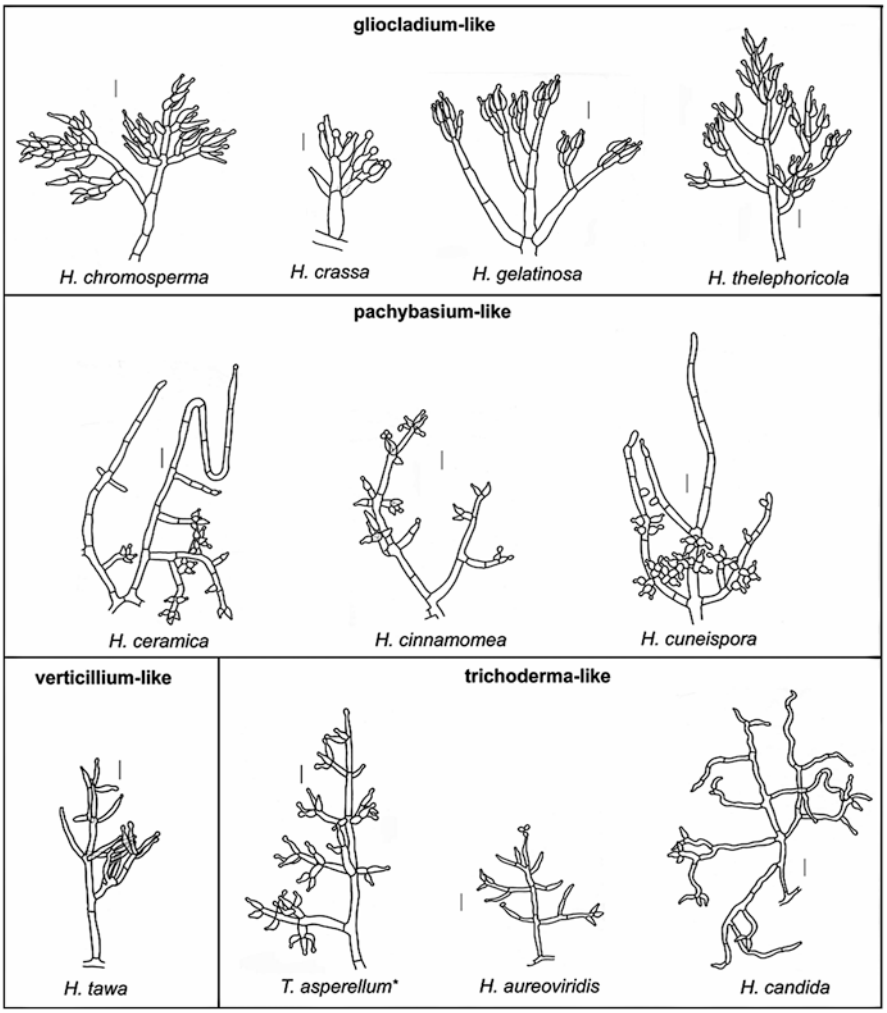


Fig. 4.3 Selected examples are showed the five pattern types of conidiosphore of *Trichoderma/Hypocrea* species which are *Acremonium*-like, *Gliocladium*-like, *Pachybasium*-like, *Verticillium*-like and *Trichoderma*-like pattern (Chaverri and Samuels 2003)

2. ***Verticillium-like*:** unbranched or slight branched erect conidiophores with verticils or whorls of distinctly divergent phialides on several levels; phialides within a whorl originate on the same level around the main axis. Contrary to true *Verticillium*, the term is used for conidiophores with secondary branches having similar arrangements.
3. ***Gliocladium-like*:** unbranched or more or less intensely branched conidiophores with penicilli of densely packed and appressed, more or less parallel, phialides at the top.

4. ***Pachybasium-like*:** conidiophores typically densely branched and stout, with small ampulliform or lageniform phialides. Part of the conidiophores are often ending in simple or branched, sterile or fertile elongations.
5. ***Trichoderma-like*:** is difficult to circumscribe, due to the lack of a strict borderline to *verticillium*-like. It comprises narrow, flexible and richly branched conidiophores that may be irregular, with distinct curvatures or terminally pyramidal with branches often at right angles. The phialides are typically lageniform, in irregular clusters, often bent, sometimes repetitive. Frequently, *verticillium*-like structures are also present on *trichoderma*-like conidiophores.

4.2.9 Effuse Conidiation (Synanamorphs)

Effuse conidiation (synanamorphs): conidiation without formation of distinct, macroscopically visible units. They are usually made up of more or less evenly distributed simple erect conidiophores, macroscopically only visible if densely arranged and forming green conidia. Effuse conidiation may also occur in macroscopically visible, downy or hairy areas on long aerial hyphae. Conidia of effuse conidiation are typically formed in wet heads.

4.2.10 Pustules

Pustules: are in their extreme form distinct dense opaque conidiation structures, often pulvinate with circular outline, originating on a single stipe, a thick aerial hypha from which primary branches emerge. Pustules may grade into transparent, loose structures, i.e., tufts.

4.2.11 Shrubs, Granules

Shrubs, granules: are small conidiation units (usually larger single conidiophores) spread on the agar like sand (granules), often scarcely visible macroscopically; 0.2–0.5 mm diam. They may constitute the final stage of conidiation or develop between effuse and pustulate conidiation.

4.2.12 Tufts, Also Called "Fluffy Tufts"

Tufts, also called "fluffy tufts": are conidiation structures appearing macroscopically as loose, cottony, often confluent masses or aggregations of conidiophores with variable outlines, usually not circular. In some species pustules develop from tufts.

4.3 Macroscopic Analysis

Several groups of *Trichoderma* colonies are analysed by morphological characteristics for identification of *Trichoderma* isolates. This procedure is allowed large number of samples making in a small timeframe and cost effective. Slants culture of *Trichoderma* isolates are aseptically recultured onto a new PDA agar in petri dish and incubated for 4–5 days at room temperature condition of 22 ± 2 °C (12 h dark and 12 h light). *Trichoderma* isolates are formed several groups using the following macroscopic criteria: growth rate, mycelium density and appearance, conidiation color, pattern and coloration of the medium. Some *Trichoderma* isolates have ability to produce sweet coconut odor.

4.3.1 Trichoderma asperellum

The macroscopic and microscopic observations of *T. asperellum* are shown in Figs. 4.4 and 4.5.

Macroscopic characteristics

- Colonies of *Trichoderma* isolates are grown on PDA for 5 days at 28 ± 2 °C in darkness with brief exposure to ambient fluorescent light (8 h) forming one to two concentric rings near the inoculum zone with a dense conidial production, with white conidia toward the green centre (Fig. 4.4(a)).
- For the colony reverse, it is creamy in color and often folded or convoluted.
- No color diffusion or pigment is observed throughout the PDA plate (Fig. 4.4(b)).
- A lack of aerial mycelium.
- Absence sweet coconut odor.

Microscopic characteristics

- The conidiosphores are highly branches and arrangement in symmetric order.
- The conidiosphores primary branches arising below the tip often paired with same length and projecting at nearly 90 degree to the main axis.
- The phialides typically produced at the tips of the primary, secondary and tertiary branches, rare straight along the length of the branches, typically in whorls of 2–4 phialides, straight, ampuliform, slightly enlarged in the middle with 8 μm long in average.
- The phialides slightly enlarged in the middle with 1.0–2.0 m width.
- Intercalary phialide has not found in these isolates.
- In addition, the dark green conidia which are globose to subglobose or ovoidal in the size of 3–4 μm observed.
- Within 1 week, the chlamydophores are formed either terminal or frequently intercalary, on immersed hyphae, subglobose to ovoidal, smooth and pale green.

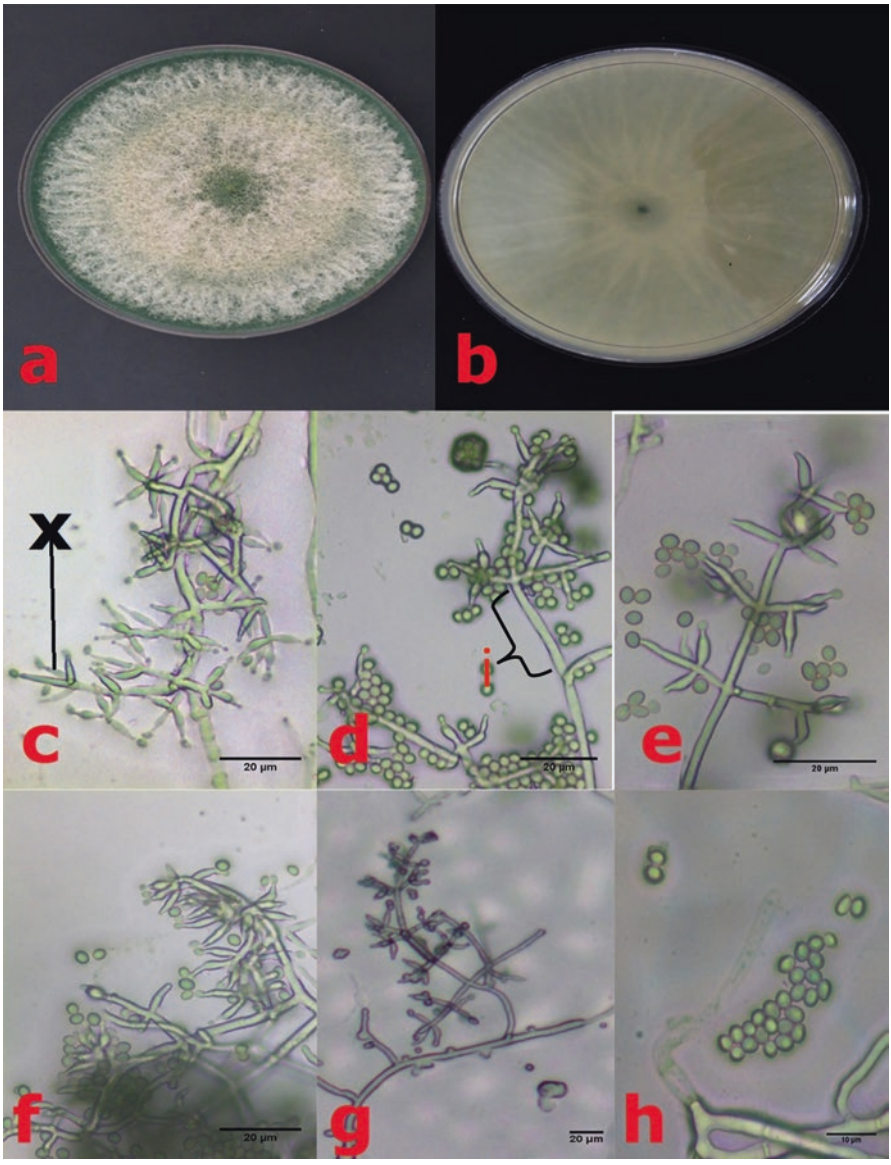


Fig. 4.4 A representative morphological characteristics of *Trichoderma asperellum*. (**a**) Front colony which was grown in PDA for 5 days; (**b**) Reverse colony; (**c–g**). Conidiosphores; (**h**) Conidia; X. Phialides. **c–f** and **h** were observed with 400× magnification while **g** was observed with 100× magnification

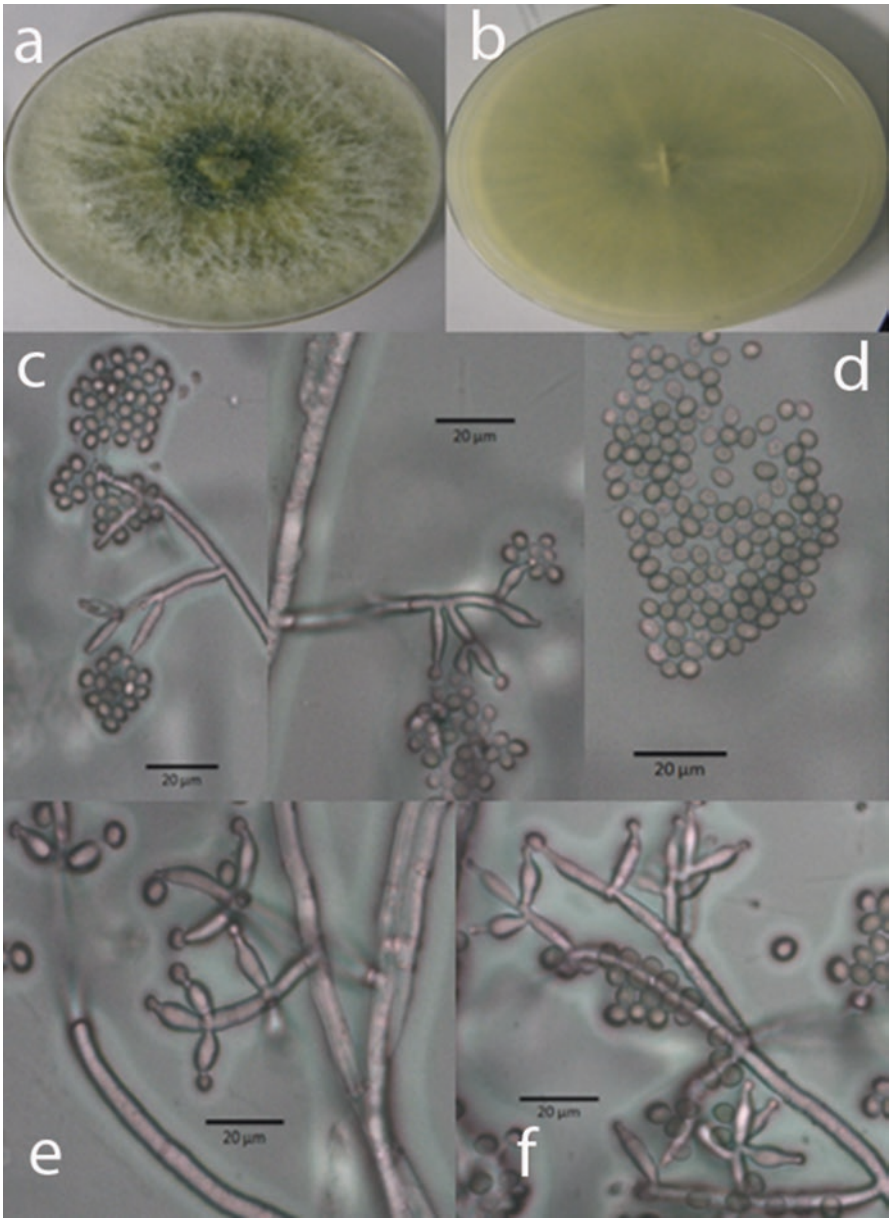


Fig. 4.5 A representative morphological characteristics of *Trichoderma asperellum*. (**a**) Front colony which was grown in PDA for 5 days; (**b**) Reverse colony; (**c**) Phialides with condia; (**d**) Conidia; and (**e**, **f**) Conidiosphores were observed with 400× magnification

4.3.2 *Trichoderma harzianum*

The macroscopic and microscopic characteristics of *T. harzianum* are shown in Figs. 4.6 and 4.7.

Macroscopic characteristics

- Colonies of *T. harzianum* isolates usually formed 1–2 concentric rings with green conidial production in mature colonies (Figs. 4.6 (a) and 4.7 (a)) during incubation for 5 days at 28 ± 2 °C.
- It has observed to cause intense coloration of the agar from yellow to dark brown pigments in mature colonies (Fig. 4.6(b)).
- The mycelium is initially smooth, watery white in color and sparse, until floccose aerial mycelium has produced.
- Conidiation has predominantly effused with pustules typically merged into large irregular masses with powdery surface appearance.
- Indistinct sweet coconut odor produced.

Microscopic characteristics

- The conidiosphores are spread to the top in pyramidal fashion and highly branches (Fig. 4.6(c–g)). The main branches mostly in groups of three or four are obtained.
- Branches of the conidiosphores are typically paired.
- Phialides are flask shaped and typically short and broad in the middle with the average length of 4–6 μm.
- The phialides are observed often terminates in a narrow neck and arising mostly in crowded and have whorls of 3–5; the whorls are typically flask-shaped and enlarged in middle.
- The length of phialides has tended to be shorter than other *Trichoderma* species with the average length of 6.0–10.00 μm.
- The conidia [Figs. 4.6 and 4.7(b)] tend to be globose to subglobose in shape with the average length of 2–3 μm and pale green in color.
- Pustules are not formed or poorly formed.

4.3.3 *Trichoderma hamatum*

The macroscopic and microscopic observations of *T. hamatum* are shown in Fig. 4.8.

Macroscopic characteristics

- The colonies grow moderately rapid, close to the agar, and entire the whole petri dishes within 3 days during incubation period at 28 ± 2 °C.
- It produced some aerial mycelium which is white in color with some yellow conidiation in the centre of the plate and grown densely (Fig. 4.8(a)).
- Isolates never cause discoloration of the agar (Fig. 4.8(b)) and odorless.

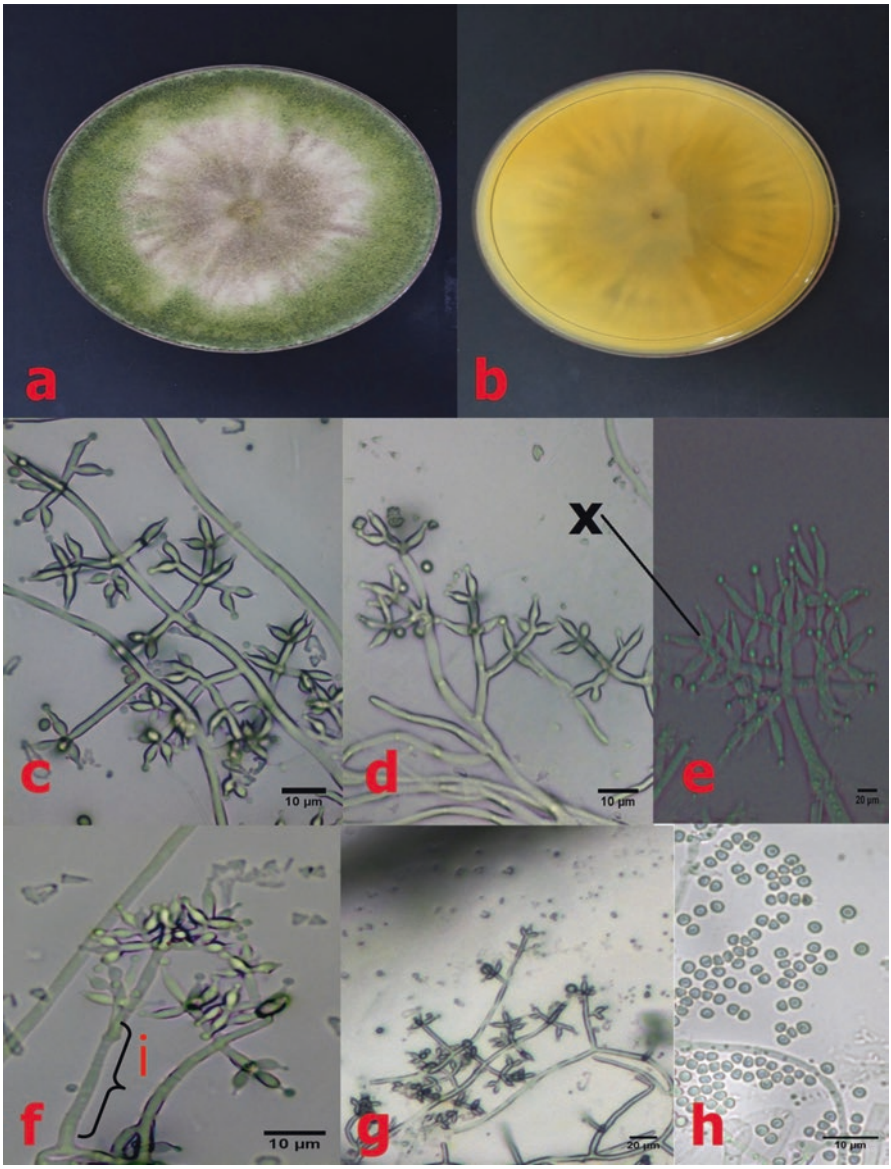


Fig. 4.6 A representative morphological characteristics of *Trichoderma harzianum.* (**a**) Front colony which was grown in PDA for 5 days; (**b**) Reverse colony; **c–g** and X. Phialides; **h** Conidia and **c–g** and i Conidiosphores were observed with 400× magnification while **g** and **h** was observed with 100× magnification

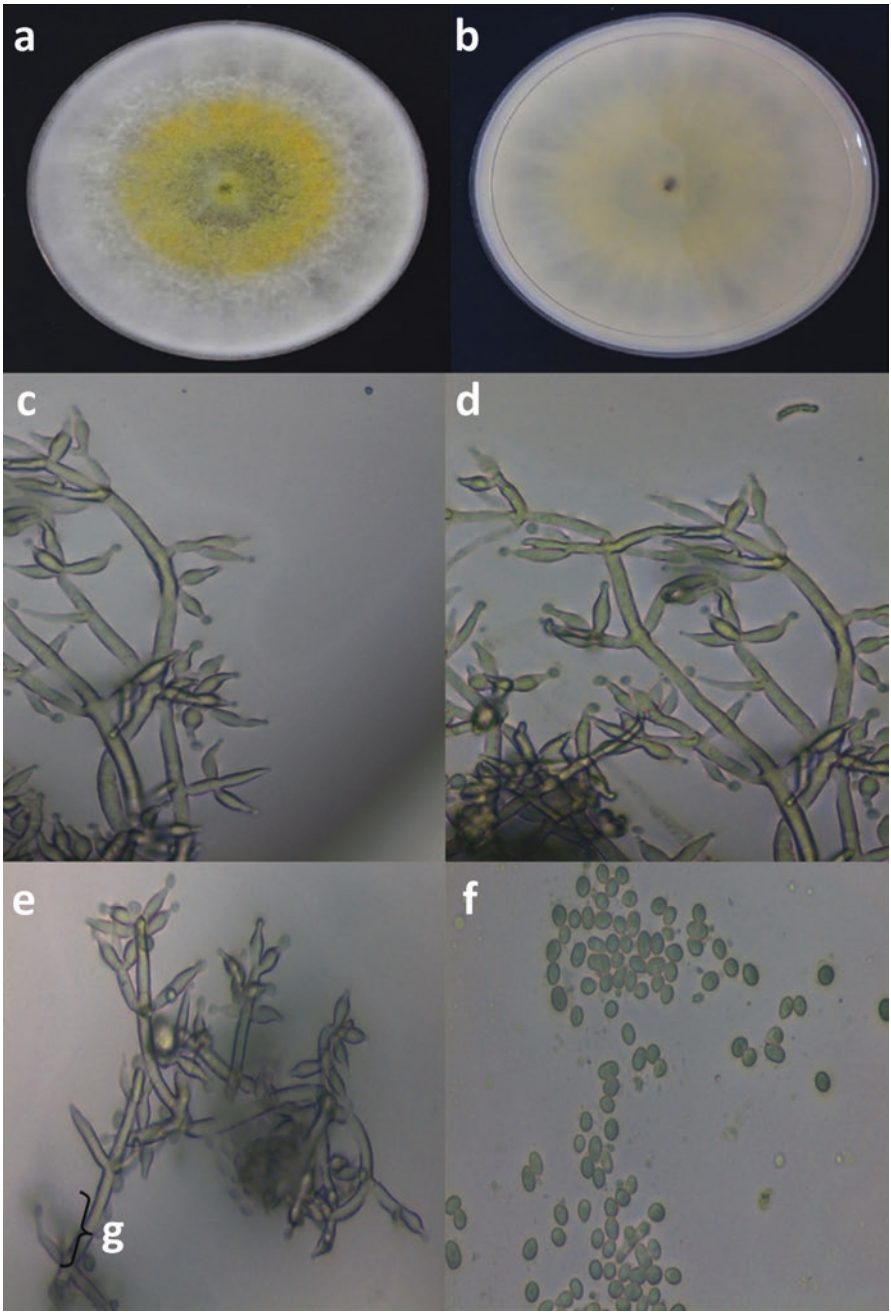


Fig. 4.7 A representative morphological characteristics of *T. harzianum.* (**a**) Front colony which was grown in PDA for 5 days; (**b**) Reverse colony; (**c–e**) Phialides; (**g**) Conidiosphores; and (f) Conidia were observed with 400× magnification while **f** was observed with 100× magnification

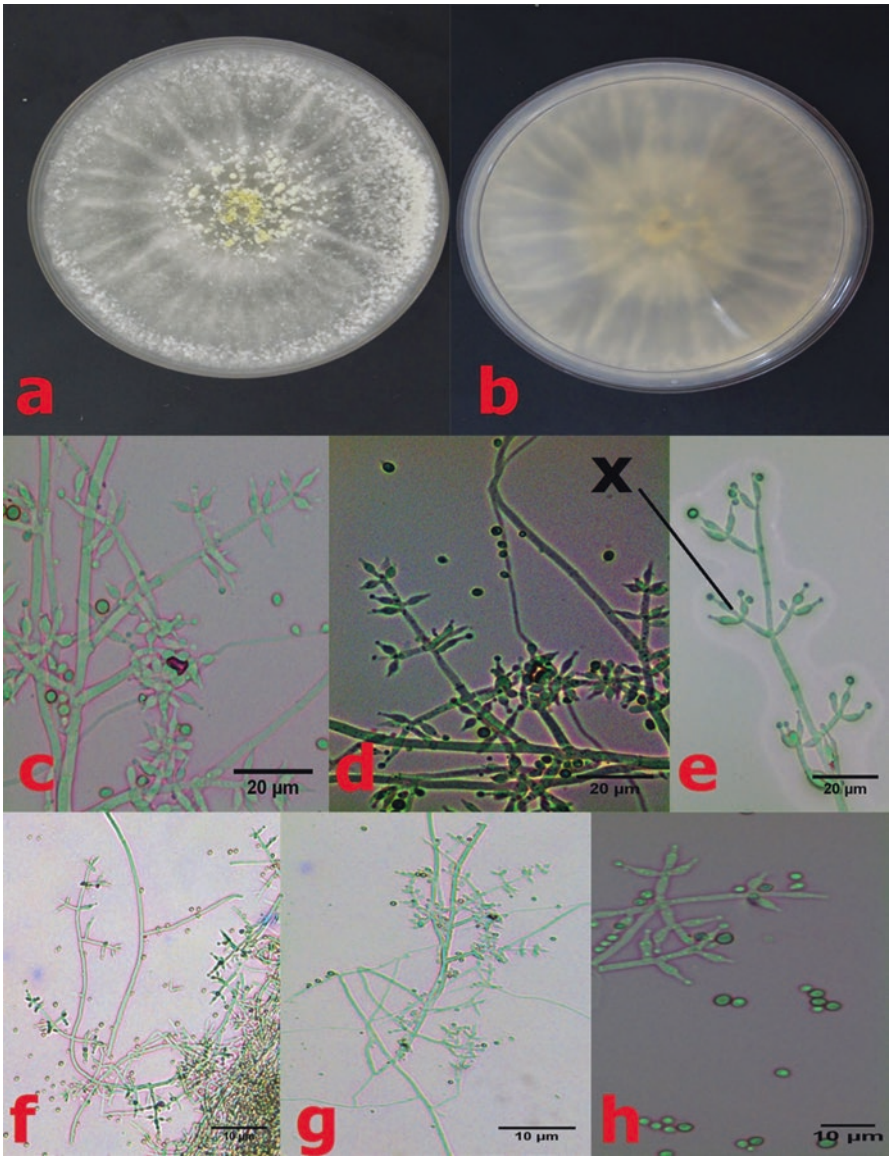


Fig. 4.8 A representative morphological characteristics of *Trichoderma hamatum*. (**a**) Front colony which was grown in PDA for 5 days; (**b**) Reverse colony; (**c–g**) Conidiosphores; (**h**) Conidia; X. Phialides. **c–e** and **h** were observed with 400× magnification while **f** and **g** were observed with 100× magnification

- The colonies produced dispersed cushion shaped, compact tufts of conidiosphores with some color variation in these isolates ranging from pale yellow, greenish yellow to greyish green.
- The conidia tend to form in concentric rings.

Microscopic characteristics

- The conidiosphores tend to aggregate in fascicles or pustules and highly branches in irregular pattern (Fig. 4.8(c–g)).
- The lateral branches usually comprised one or few broad cells with phialides arising at the tip and along the length. While the secondary branches may arise as single cells which phialides.
- The phialides are formed from short lateral branches at the base of the elongation, mostly ellipsoidal to ovoidal with an average length of 5–7 μm long, 3–4 μm wide at the widest point.
- Phialides arising in 1–3 whorls and densely clustered on board.
- Intercalary phialides are absent in these isolates.
- The conidia have a smooth walled, ellipsoidal, and green in color.
- The average length of the conidia obtained from 4 to 5 μm (Fig. 4.8(h)).
- Absence of chlamydospores.

4.3.4 Trichoderma reesei

The macroscopic and microscopic observations of *T. reesei* are shown in Fig. 4.9.

Macroscopic characteristics

- The colonies grow rapidly on PDA in room temperatures condition of 28 ± 2°C and produce an intense diffusing yellow pigment and fewer yellowish green conidia as they tend to form on the centre of the plate (Fig. 4.9(a, b)).
- One or two concentric rings may appear (usually in old culture), one near the margin and the other around the inoculum point.
- Apart from, cottony aerial mycelium is not forming.
- No distinctive odor has noticed from the isolates.

Microscopic characteristics

- The conidiosphores are sparingly branches (Fig. 4.9(c–g)). The primary branch is long while the secondary branch usually short and paired branching systems are rare.
- Each branch terminating in one or two phialides which are arising singly from the main axis and intercalary phialides are commonly observed in Fig. 4.9(f).
- Phialides are cylindrical or slightly inflated with an average length of 5–8 μm.
- The conidia are smooth walled, pale green in color and oblong or ellipsoidal in shape with an average length of 3–5 μm (Fig. 4.9(h)).

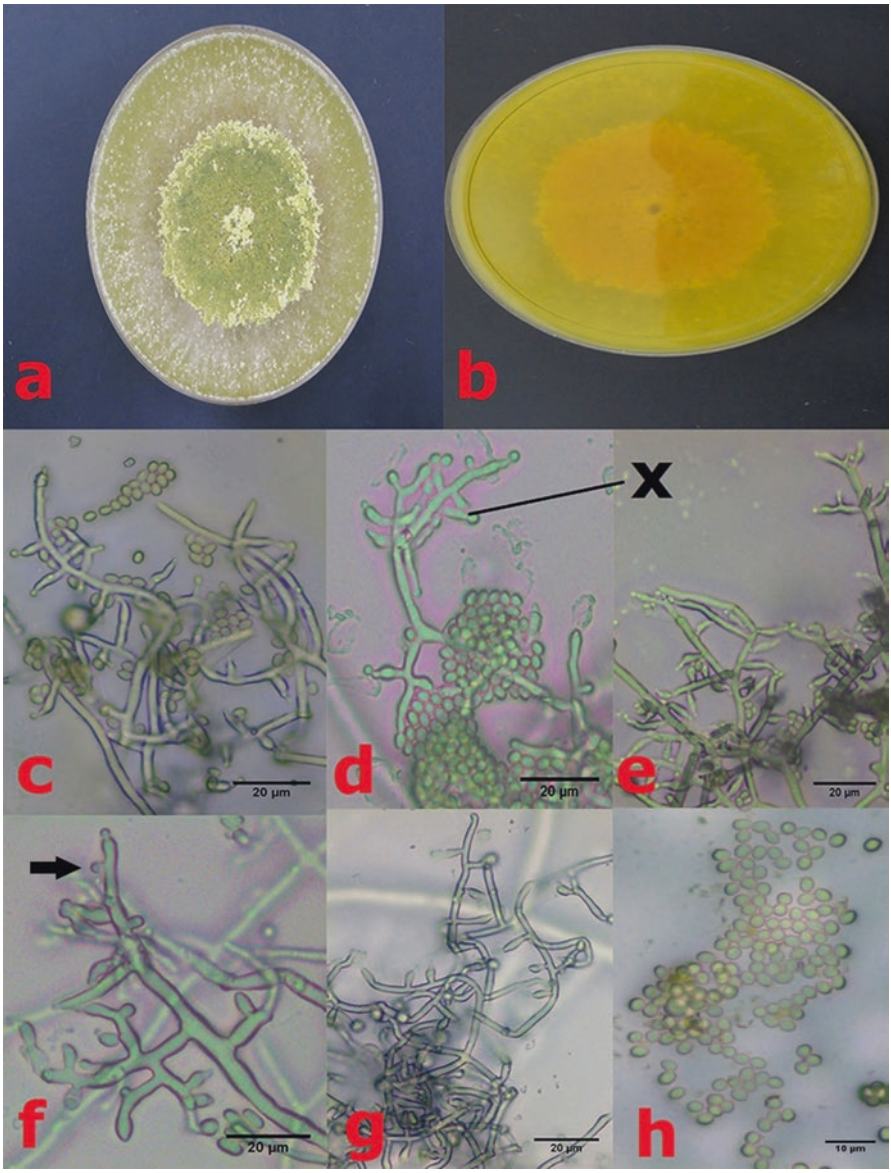


Fig. 4.9 A representative morphological characteristics of *T. reesei*. (**a**) Front colony which was grown in PDA for 5 days; (**b**) Reverse colony; (**c–g**) Conidiosphores; (**h**) Conidia; *Arrow*: Intercalary phialides; X: Phialide. **c–h** were observed with 400× magnification

4.3.5 *Trichoderma longibrachiatum*

The macroscopic and microscopic characteristics of *T. longibrachiatum* are shown in Fig. 4.10.

Macroscopic characteristics

- The colony is initially smooth, glabrous, translucent and watery white but often to form greenish yellow conidial crusts with dense conidiation (Fig. 4.10(a)) when grown onto the PDA for 5 days at 28 ± 2 °C with a good interval of light and darkness.
- The reverse colony can observe to be pale greenish-yellow in young culture (Fig. 4.10(b)) to brownish chocolates in mature culture.
- No distinct odor appearance.

Microscopic characteristics

- The conidiosphores are typically long and the phialides are bottle shape, mostly solitary and often inflate in the middle.
- The phialides often observe to bent at the apex, and slightly constrict at the base with an average length around 6–9 μm (Fig. 4.10(c–g)).
- They arise singly from the main axis and intercalary phialides commonly produced as shown in Fig. 4.10(c).
- Conidia are smooth walled with ellipsoidal to cylindrical in shape with an average length of 3–6 μm.
- The color of the conidia has observed to be pale green (Fig. 4.10(h)). Sometime a smooth, thick-walled and subglobose to ellipsoidal.
- Chlamydospores can be observed in mature colony.

4.3.6 *Trichoderma spirale*

The morphological characteristics of *T. spirale* are shown in Fig. 4.11.

Macroscopic characteristics

- The colonies grow well onto PDA at 28 ± 2 °C with a less distinctive concentric rings observed. The conidiation of the conidia appear to be compact, cottony, cushion-shaped pustules and the mycelia appear to be white with greenish yellow tint in color (Fig. 4.11(a)).
- A yellow pigment tends to diffuse throughout the agar.
- Reverse colony is colorless; however most of the isolates are developing a dull-yellowish color (Fig. 4.11(b)).
- No distinctive odor produced.

Microscopic characteristics

- The conidiosphores observed to be long and less branches.
- The branches are relatively short and arising singly, alternately or pair from the main axis (Fig. 4.11(c–g)).

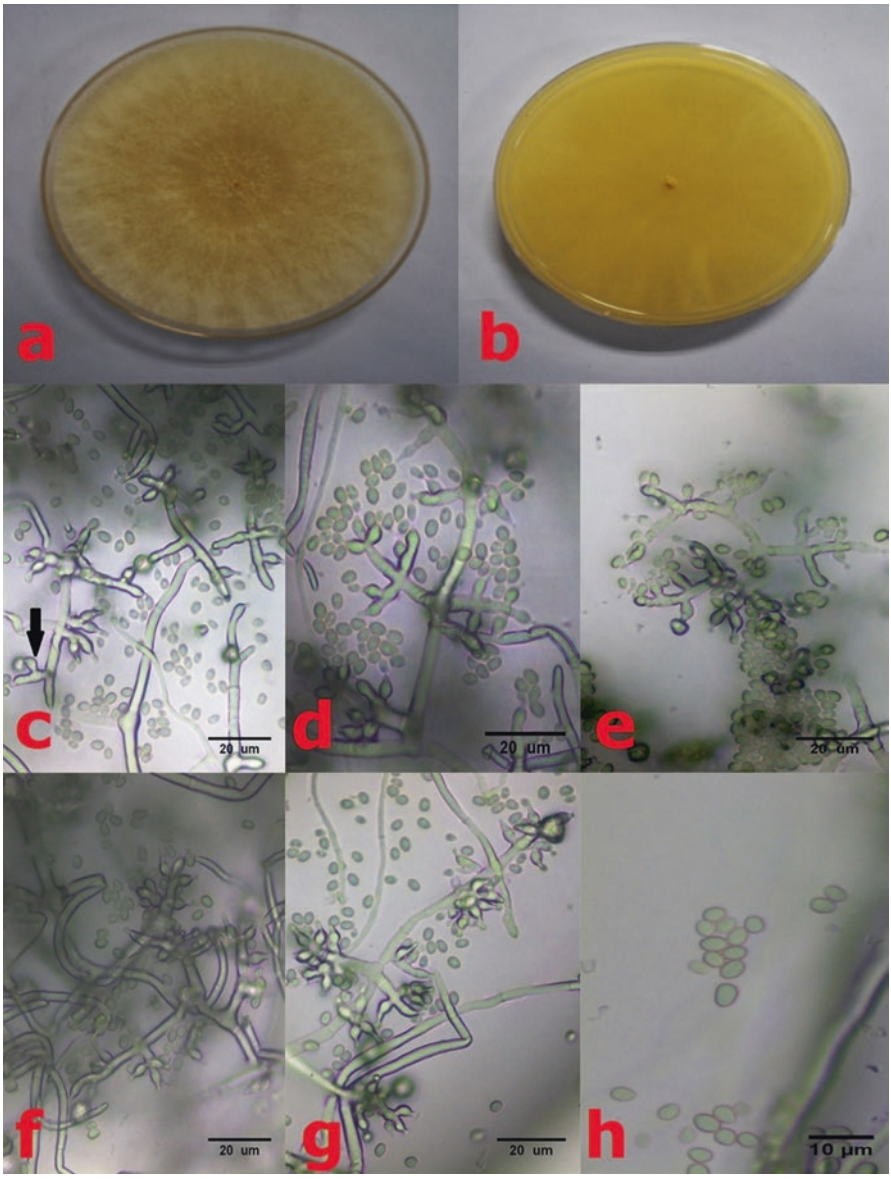


Fig. 4.10 Morphological characteristics of *T. longibrachiatum*. (**a**) Front colony which was grown in PDA for 5 days; (**b**) Reverse colony; (**c–g**) Conidiosphores; (**h**) Conidia; *Arrow*: Intercalary phialides. **c–h** were observed with 400× magnification

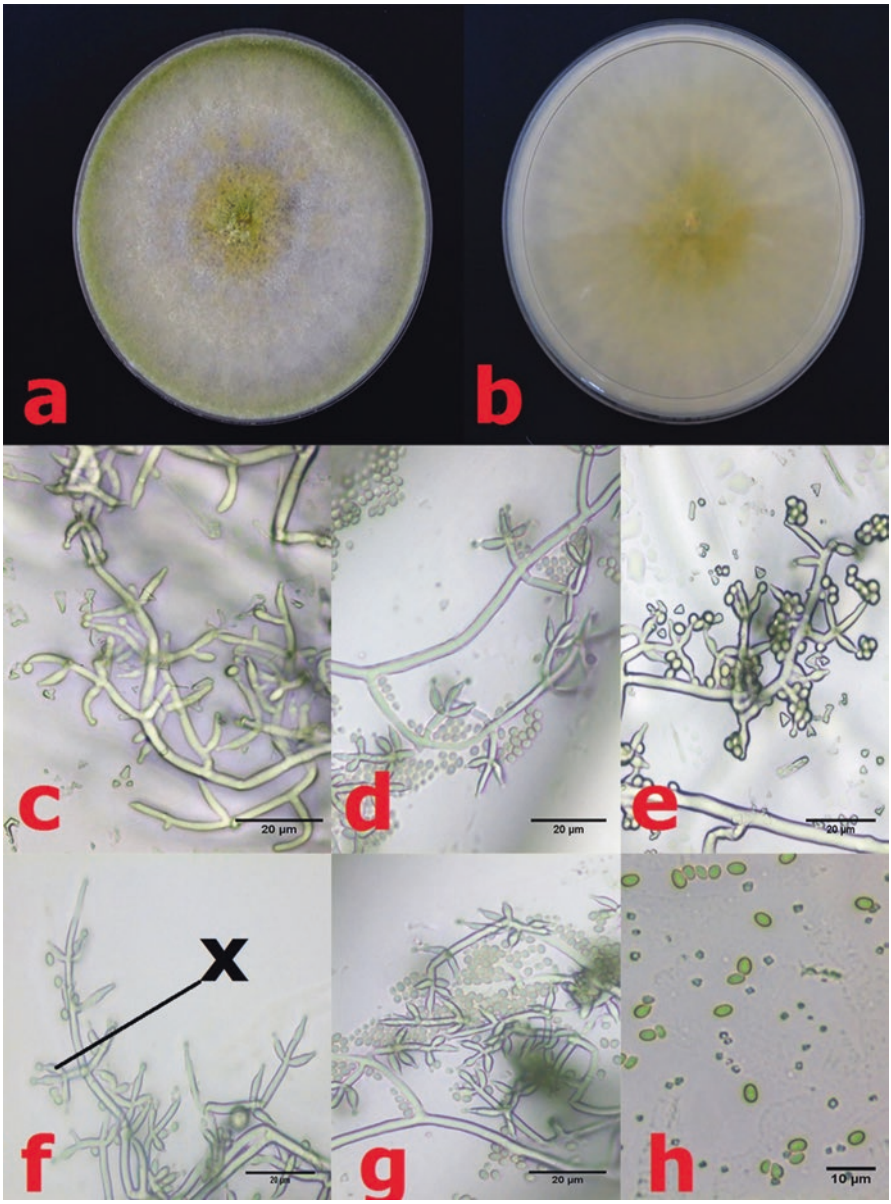


Fig. 4.11 A representative morphological characteristics of *T. spirale*. (**a**) Front colony which was grown in PDA for 5 days; (**b**) Reverse colony; (**c–g**) Conidiosphores; (**h**) Conidia; X: phialides. **c–h** were observed with 400× magnification

- The primary branches are terminated with fertile elongation (Fig. 4.11(f)).
- Phialides appear to be ampulliform to nearly subglobose which an average length of 4–6 µm. Most of the phialides arising in dense cluster comprising 1–3 whorls.
- Absence of intercalary phialide.
- The conidia observe to be green in color, smooth walled, and oblong to ovoidal with an average length of 3–5 µm.

4.3.7 *Trichoderma brevicompactum*

The morphological characteristics of *T. brevicompactum* are shown in Fig. 4.12.

Macroscopic characteristics

- The colony grows moderately slow at 28 ± 2 °C, getting upto 7 cm in diameter during 72 h of incubation period in PDA plate (Difco, USA) as compared to other isolates.
- After 5 days of incubation under intermittent light, the colony can form conspicuous 1–2 concentric rings with dense yellow-green conidia over the internal concentric ring (Fig. 4.12(a)).
- There is no diffuse pigment occur throughout the agar (Fig. 4.12(b))
- Absence of distinctive odor.

Microscopic characteristics

- The conidia can observe to hyaline and smooth-walled under 400× magnification.
- The branching pattern of the conidiosphores resembles the branching pattern of the *Pachybasium*-type (Fig. 4.12(c–f)).
- Each branches of the conidiosphores terminating in a vertical of 1-3 phialides.
- Phialides appear to be marginally enflamed in the middle and lageniform when arising from crowded branches.
- Absence of intercalary phialide.
- The conidia are smooth, subglobose or short ellipsoidal and yellowish green on color with an average length of 2-3 µm (Fig. 4.12(h)).

4.3.8 *Trichoderma erinaceum*

Trichoderma erinaceum which belongs to the section of *Viride* based on the morphological characteristics are shown in Fig. 4.13.

Macroscopic characteristics

- Conidia can form in dense, flat lawns in concentric rings with some tendency to form flat or infrequently develop pustules at 25–30 °C.
- Diffusing pigments and distinctive odor are absence.

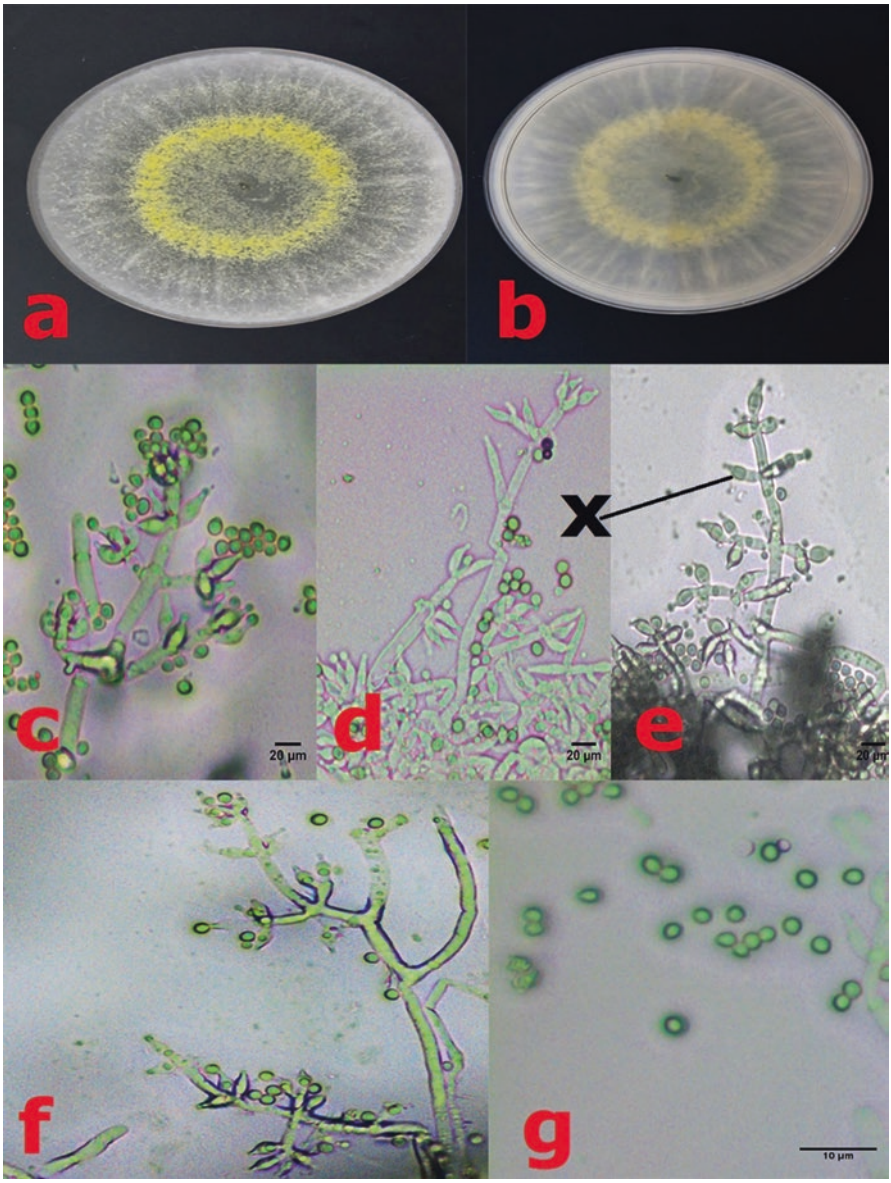


Fig. 4.12 Morphological characteristics of *T. brevicompactum*. (**a**) Front colony which was grown in PDA for 5 days; (**b**) Reverse colony; (**c–f**) Conidiosphores; (**g**) Conidia; X: phialides. **c–g** were observed with 400× magnification

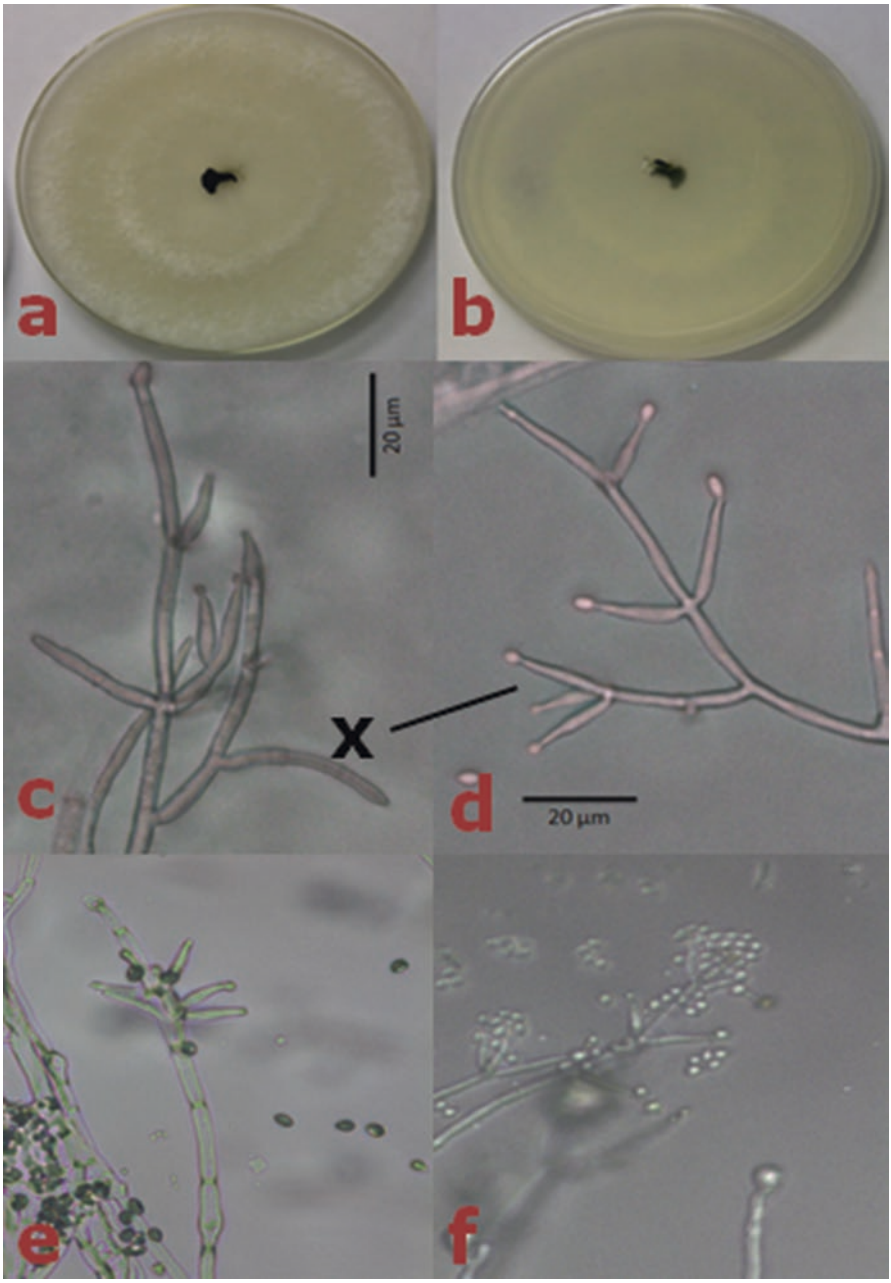


Fig. 4.13 A representative morphological characteristics of *T. erinaceum*. (**a**) Front colony which has grown in PDA for 4 days; (**b**) Reverse colony; (**c–f**) Conidiosphores; X. Phialides. **c–f** were observed with 400× magnification.

Microscopic characteristics

- The conidiosphores branches arising at angles of 90° or less with the main axis.
- The main axis terminates in septate elongation with single phialide at its tip.
- The phialide arising from the branches near the base solitary or in whorls of 2 or 3, straight, nearly cylindrical to swollen in the middle as can be seen in Fig. 4.13. Conidiosphores branches are arising at the angle of 90° directly or not to the conidiosphores with the average of 2.2–3.0 μm wide.
- Chlamydospores are terminally intercalary, globose to subglobose.

4.3.9 Trichoderma koningiopis

The macroscopic and microscopic characteristics of *T. koningiopis* are observed in Figs. 4.14 and 4.15.

Macroscopic characteristics

The representative's morphological characteristics of *T. koningiopsis* are shown in Figs. 4.14 and 4.15.

- The conidia start to grow after 2 days incubation at 28 ± 2 °C.
- It has dense lawn or the centre remaining sterile, forming 2–3 concentric rings, abundant in light-grown colonies, and less abundant.
- No discoloration seen on the agar.
- Distinctive odor not detected.

Microscopic characteristics

- The conidiosphores abundant branches arising along the length of the main axis, pair with longer or shorter internodes between branches with *pachybasium*-like pustules, short and crowded phialides.
- Branches arise at the angle less than 90°.
- Phialides are straight, hooked or sinuous, narrowly lageniform and sometime obviously swollen in the middle.
- Several phialides are arising from the same appoint and crowded.
- The conidia are deep to dark green, seldom with yellow coloration, ellipsoidal, lacking visible basal abscission scar and smooth.
- Chlamydospores are fertile to sparse, terminal to intercalary, globose to subglobose with the range of 9.0–9.5 μm.

4.3.10 Trichoderma melanomagna

Trichoderma melanomagna which belongs to the section of *Lutea* based on the morphological characteristics as shown in Fig. 4.16.

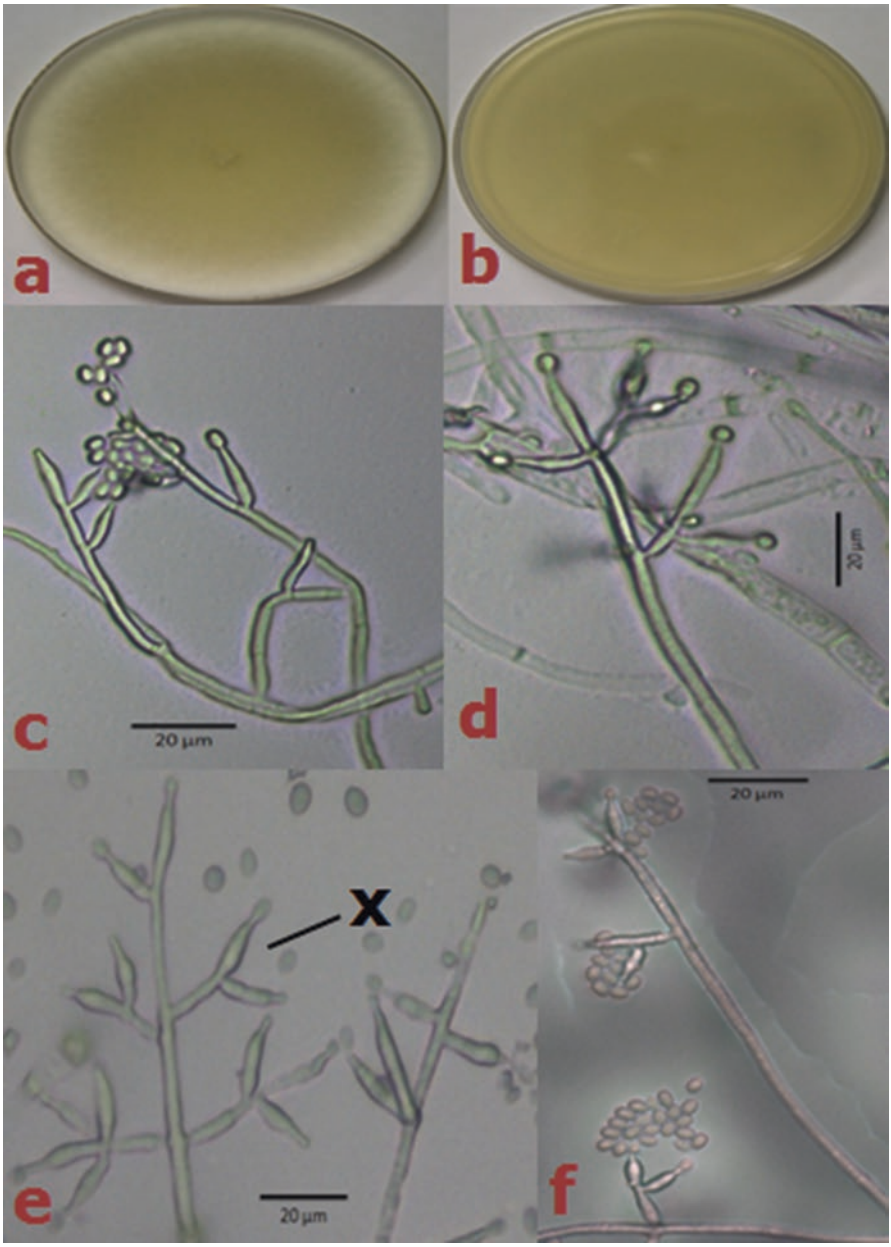


Fig. 4.14 A representative morphological characteristics of *T. koningiopsis*. (**a**) Front colony which has grown in PDA for 4 days; (**b**) Reverse colony; (**c–f**) Conidiosphores; X. Phialides. **c–f** were observed with 400× magnification

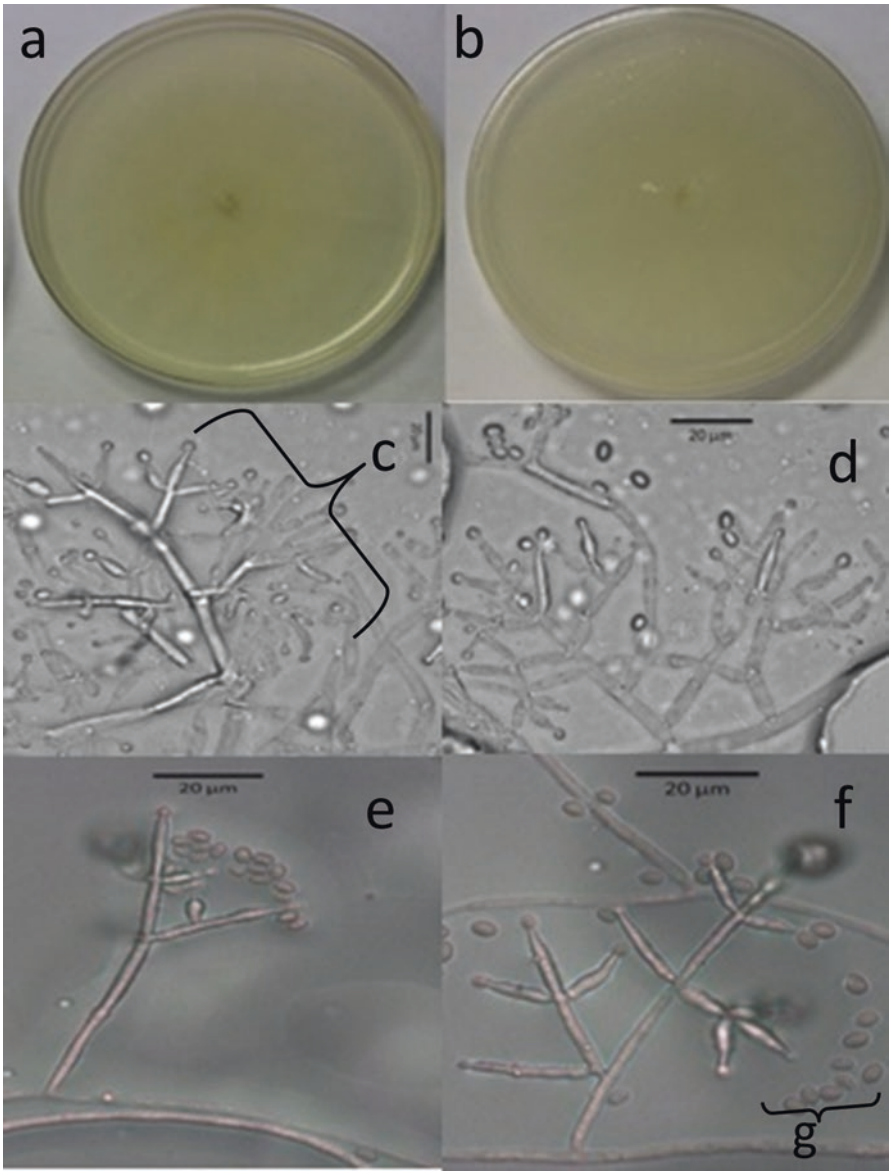


Fig. 4.15 A representative morphological characteristics of *T. koningiopsis*. (**a**) Front colony which has grown in PDA for 4 days; (**b**) Reverse colony; (**d–f**) Conidiosphores; (**c**) Phialides and (**g**) Conidia (observed with 400× magnification)

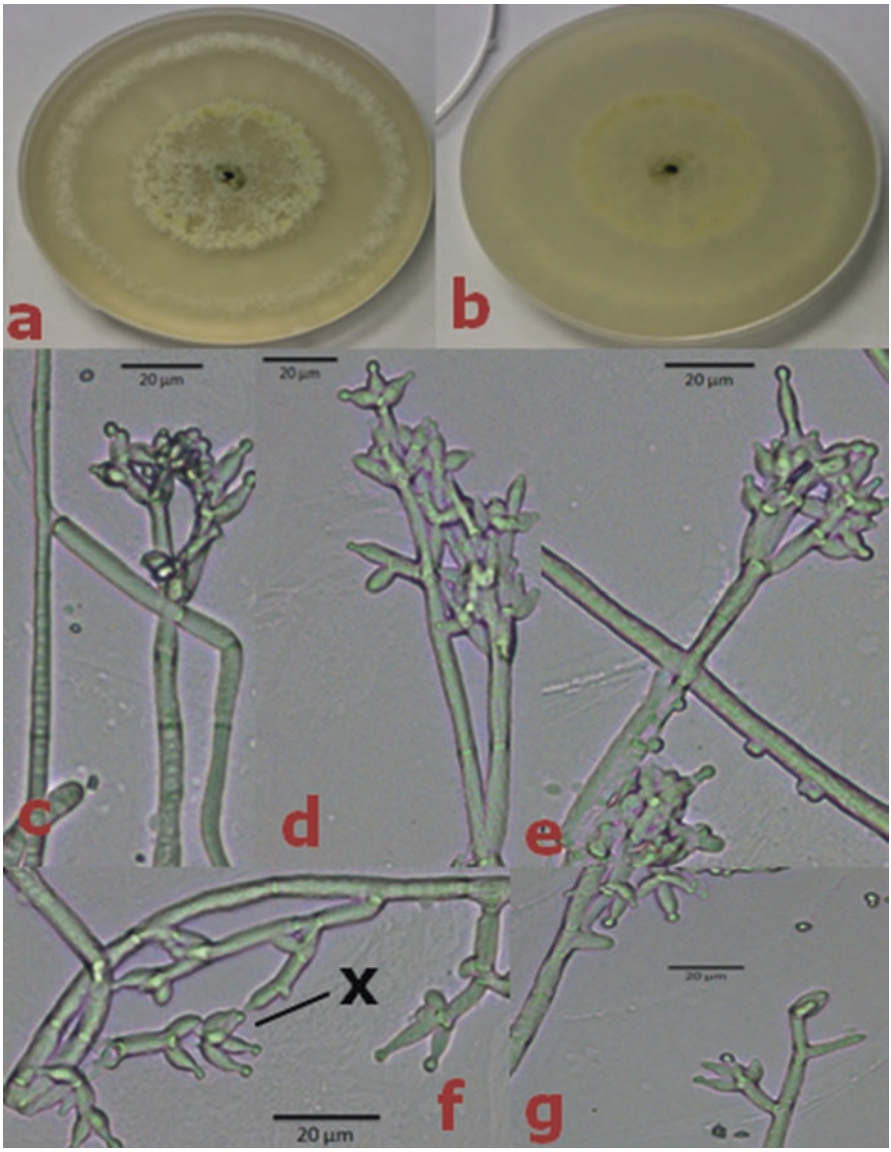


Fig. 4.16 A representative morphological characteristics of *T. melanomagna* (**a**) Front colony which has grown in PDA for 4 days; (**b**) Reverse colony; (**c–f**) Conidiosphores; X. Phialides. **c–f** were observed with 400× magnification

Macroscopic characteristics

- Colonies grow onto PDA rapidly at 25 °C with radius of 35 mm after 3 days.
- No distinctive odor detected.
- The production of diffusing pigment was not commonly observed.
- However, some pigments appeared, it often inconsistently formed within individual.

Microscopic characteristics

- The conidiosphores have *pachybasium*-like morphology with irregular branching pattern.
- The main axis terminates in a sterile or terminally fertile extension.
- The branches tend to be paired or in whorls, however, unilateral branching commonly found. Branches often not uniformly spaced and "pyramidal" shape in the central to the traditional form concept of *Trichoderma* has not apparent.
- Phialides are longer and slender ranging between 0.8–1.5 × 3–4 μm, in whorls with 3–4 of each phialides axis at angle of 90° to its nearest neighbour.
- Stromatal size ranges from 0.5 to 15.0 mm diameter with shades in white, mostly yellowish, orange, brown, green or black in color.
- Most stromata are pulvinate with a broad or narrow base, peltate or almost cylindrical.

4.3.11 Trichoderma viride

The morphological characteristics of *T. viride* are shown in Fig. 4.17.

Macroscopic characteristics

- The mycelium and conidia formed concentric rings (Fig. 4.17).
- The colony grown rapidly at 25 °C.
- No discoloration found.
- Occasionally the coconut odor found or sometime no distinctive odor detected.

Microscopic characteristics

- The conidiosphores typically comprised abundant central axis or the central axis 100–150 μm long and flexuous.
- The branches can pair or some not, arising at the angle at or near 90° with respect to its supporting branch.
- Sometime lateral branch can widely spaced intervals.
- Phialides are singly arising from the main axis or in whorls at 2–3 at the tip of the lateral braches or at the tip of the conidiosphores.
- It can cylindrical and sometime swollen in the middle with elongated neck, straight, sinuous. The central axis is the average of 2.2–3.2 μm wide.
- The pustules diameter of 0.5–1.0 mm, hemispherical, uniformly cottony and terminally projected fertile conidiosphores can observe.
- Chlamydospores are globose to subglobose with 8.5–1–0.5 μm diameter.

4.3.12 Trichoderma theobromicola

The macroscopic and microscopic characteristics of *T. theobromicola* are shown in Fig. 4.18. *T. theobromicola* which belongs to the section of *Pachybasium* based on the morphological characteristics.

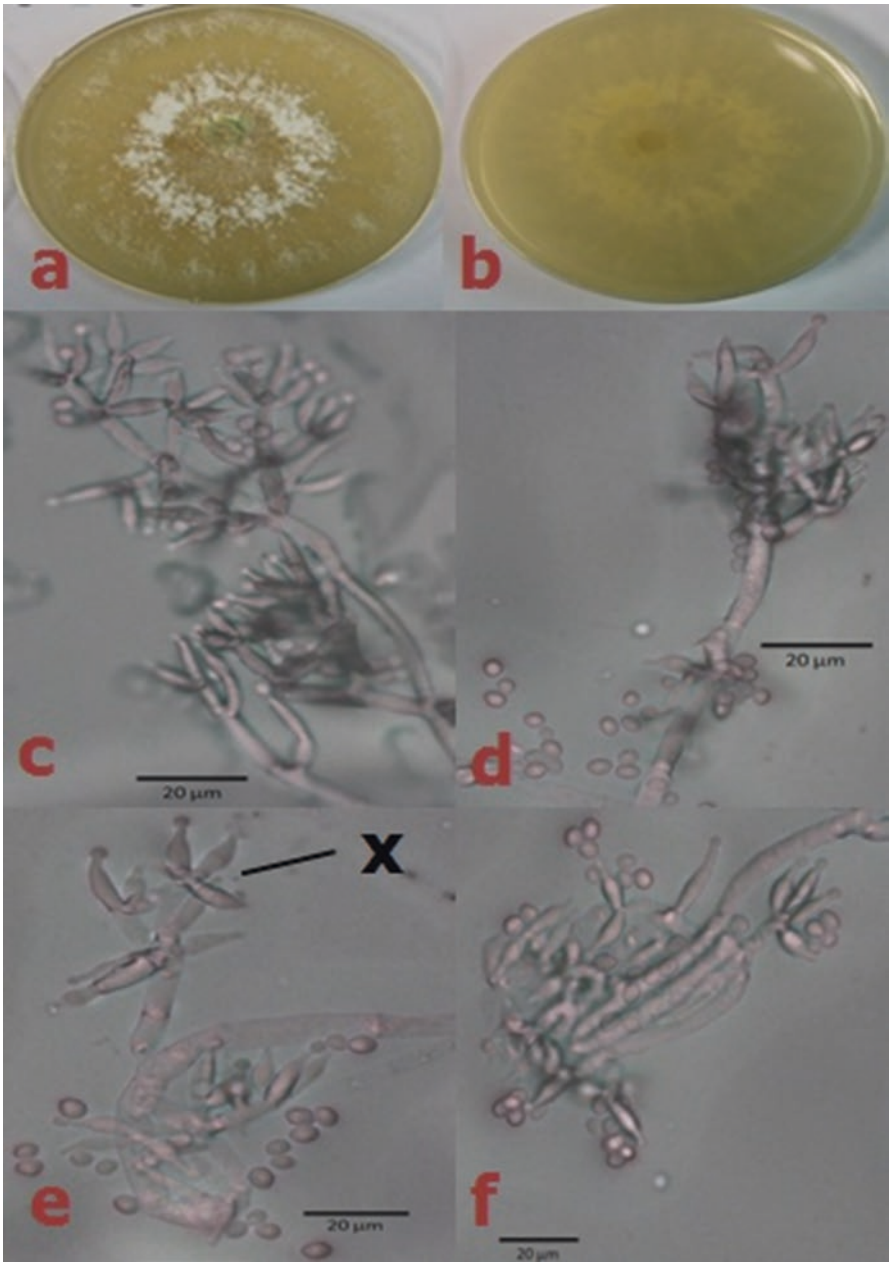


Fig. 4.17 A representative morphological characteristics of *T. viride*. (**a**) Front colony which has grown in PDA for 4 days; (**b**) Reverse colony; (**c–f**) Conidiosphores; X. Phialides. **c–f** were observed with 400× magnification

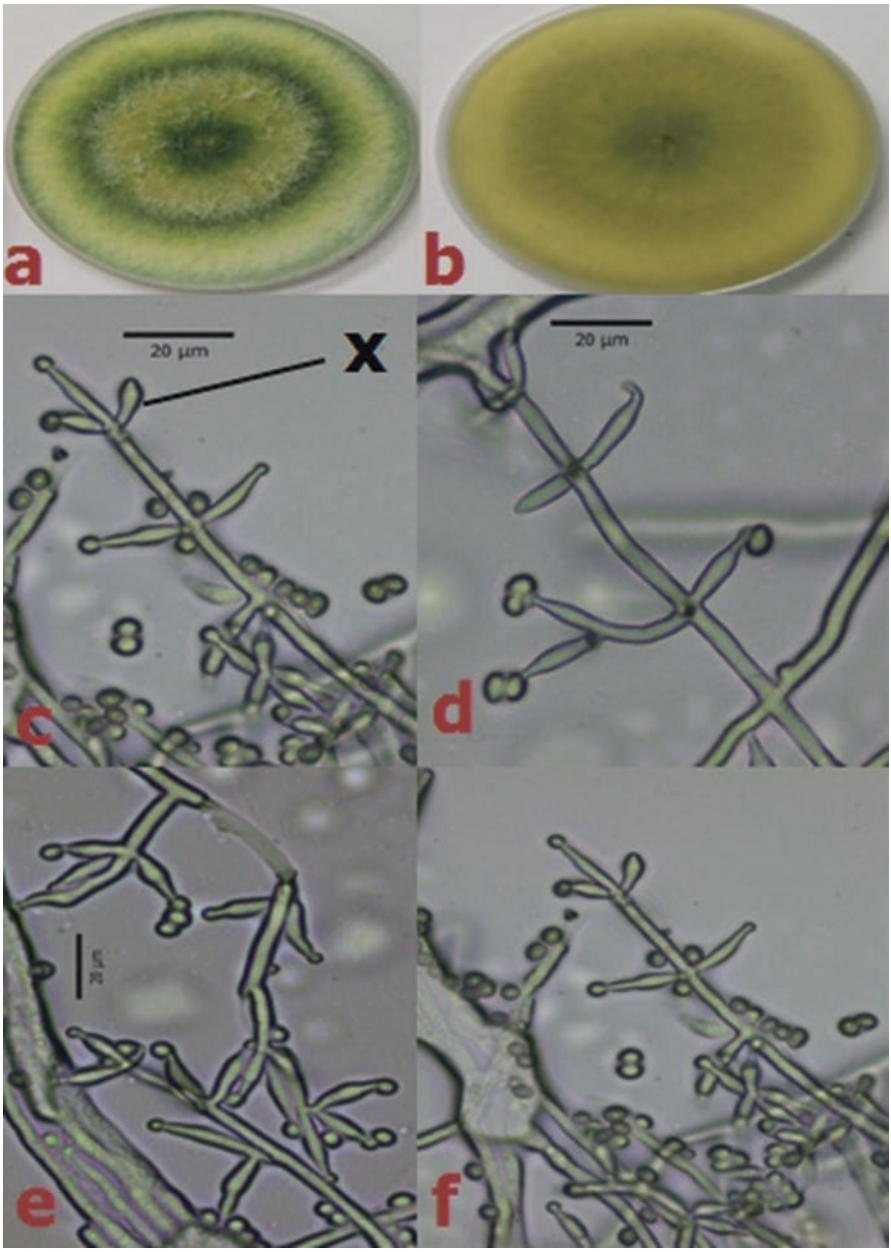


Fig. 4.18 A representative morphological characteristics of *T. theobromicola*. (**a**) Front colony which has grown in PDA for 3 days; (**b**) Reverse colony; (**c–f**) Conidiosphores; X. Phialides. **c–f** were observed with 400× magnification

Macroscopic characteristics

- Its grow rapidly onto PDA at 25 °C after 4 days of incubation, the growth of isolates colony found in the range of 50–65 mm on plates.
- It produced strong aromatic odor of coconut.
- No distinctive yellow pigment appeared.

Microscopic characteristics

- *T. theobromicola* is easily recognized based on its abundantly produced green conidia which formed in dense and thick lawns.
- It is mostly ellipsoidal to subglobose and unicellular.
- They can regularly be branched with two branches tending to arise at each node; with short internodal distances.
- The pustules are densely aggregated and papillate.
- Phialides are long and slender with the range of 1.8–3.4 μm, *gliocladium*-like at the tips of branches of the conidiosphores (Samuels et al. 2006).

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Chapter 5
Molecular Characteristics of *Trichoderma* Strains

5.1 Molecular Analysis

Morphology-based taxonomy is possible to identify accurately the genus level, but the species level identifications are exactly complicated, subjective, laborious, and lead to erroneous identification/classification. The traditional methods defined the overestimate species, while those in the mycelia appearance or those that have slow growth in culture are mostly overlooked (Davet and Rouxel 1997). The culturable isolates can utilize the energy source under the physical and chemical limitations of the growth medium. The taxonomy classification of *Trichoderma* has limitations until today. Many *Trichoderma* species have similar physical appearance under cultural conditions and show similar morphological characters, although they are completely different species. Handling of these cultures is time-consuming and laborious when a large number of isolates are used. Following these practices, the possibility of culture contamination is high, especially the fast-growing fungi always dominating slow-growing fungi inside the medium. So it is recommended that multiplications approaches should be applied for the accurately identification of *Trichoderma* isolates at the species level.

Molecular data have rehabilitated systematic and phylogenetic analyses that are consistently used in systematic laboratories. Most of the journals are reported that Gene sequencing are deposited in accessible repositories, such as a GenBank. However, as more molecular data become available so there is making more concern about the taxonomic origin of these data (Peterson et al. 2007; Nilsson et al. 2006). Although, some taxa act to be well-organized and easy to differentiate, others can only identify by specialists of microbiologist. Currently, large numbers of gene sequencing data are deposited in GenBank database that are mostly mistakenly labeled, unless eased and continuously to be associated with the wrong taxa. Sequencing based identification of *Trichoderma* isolates at the species level has several drawbacks. It is mostly involved the submission of sequences to NCBI

S. Siddiquee, *Practical Handbook of the Biology and Molecular Diversity of Trichoderma Species from Tropical Regions*, Fungal Biology,
DOI 10.1007/978-3-319-64946-7_5

BLAST and the correct identification of *Trichoderma* species on the basis of the "best hit" or a high degree of sequence similarity (e.g., >98%). Druzhinina et al. (2005) mentioned that this approach is still now prone to mistake because (1) the deposited sequences into GenBank do not maintain a quality control and various data have been found wrongly labeled species names; (2) high correspondence (or not) of a given sequence neither confirms nor refutes species identity unless the intraspecific variability of this sequence is known; and (3) even if it is known that the given species may show nt variation (e.g., 1%), this may not apply for the total sequence area, and nts in some positions may nevertheless be absolutely invariable. All this suggests that the use of molecular sequence information for species identification without phylogenetic analysis depends on the investigators experience and the number of sequences and strains that have been seen.

Druzhinina et al. (2005) have developed a rapid DNA oligonucleotide barcode for the species identification of *Trichoderma/Hypocrea* based on a library of short and species-specific gene sequences from ITS 1 and 2 loci. They introduced the first online program using oligonucleotide barcodes: *Trich*OKEY v. 1.0, available at the website of the International Subcommission on *Trichoderma* and *Hypocrea* Taxonomy (http://www.isth.info/). The library of corresponding barcodes has stored in a MySQL database, which comprises all hallmark combinations, anchors, clade specific hallmarks, and also type of sequences for each species. For the development of the barcode, 979 sequences of 88 vouchered species are representing a total of 135 ITS1 and 2 haplotypes. Figure 5.1 demonstrates the simplified algorithm of *Trich*OKEY v. 1.0. The user sequence (=input data) is first checked for the adequacy and absence of inappropriate symbols, and then annotated in the database. Consequently, the program examines for the set of *Hypocrea/Trichoderma* GHMs to verify that the submitted sequence belongs to the sequence of the genus.

Firstly, genus-specific hallmarks are determined based on ITS1 and 2 sequences that are shared by all known species of *Hypocrea/Trichoderma*, but only different in closely related genera of fungi. The constructed library of genus-, clade- and species-specific hallmarks has been deposited in the MySQL database (http://www.mysql.com) and integrated in the barcode sequence identification program *Trich*OKEY v. 1.0 accessible at www.isth.info. *Tricho*BLAST enables to identify of *Trichoderma* isolates based on the five most frequently used phylogenetic markers: (1) ITS1 and 2, (2) tef1 fourth intron, (3) tef1 fifth intron, (4) tef1 sixth exon, and (5) rpb2. As the reference databases of *Trich*OKEY and *Tricho*BLAST (ISTH 2008) are constantly updated according to the latest findings, these online search tools allow the identification of *Trichoderma* isolates or suggest them as representatives of potentially new species.

Taxonomic relationship between species, genomic sequences of one or several genes are analyzed such as internal transcribed spacers (ITS 1, 5.8S, and ITS 2) of the nuclear ribosomal RNA gene repeat, a portion of the translation elongation factor 1-α (*tef-1*) gene including one intron, a portion of the actin (*ACT*) gene including one intron, and a portion of the calmodulin (*CAL*) gene including two introns. In my opinion, an essential link between data and taxa can provide a means to verify the taxonomic characters of the *Trichoderma* isolates sequenced and morphological

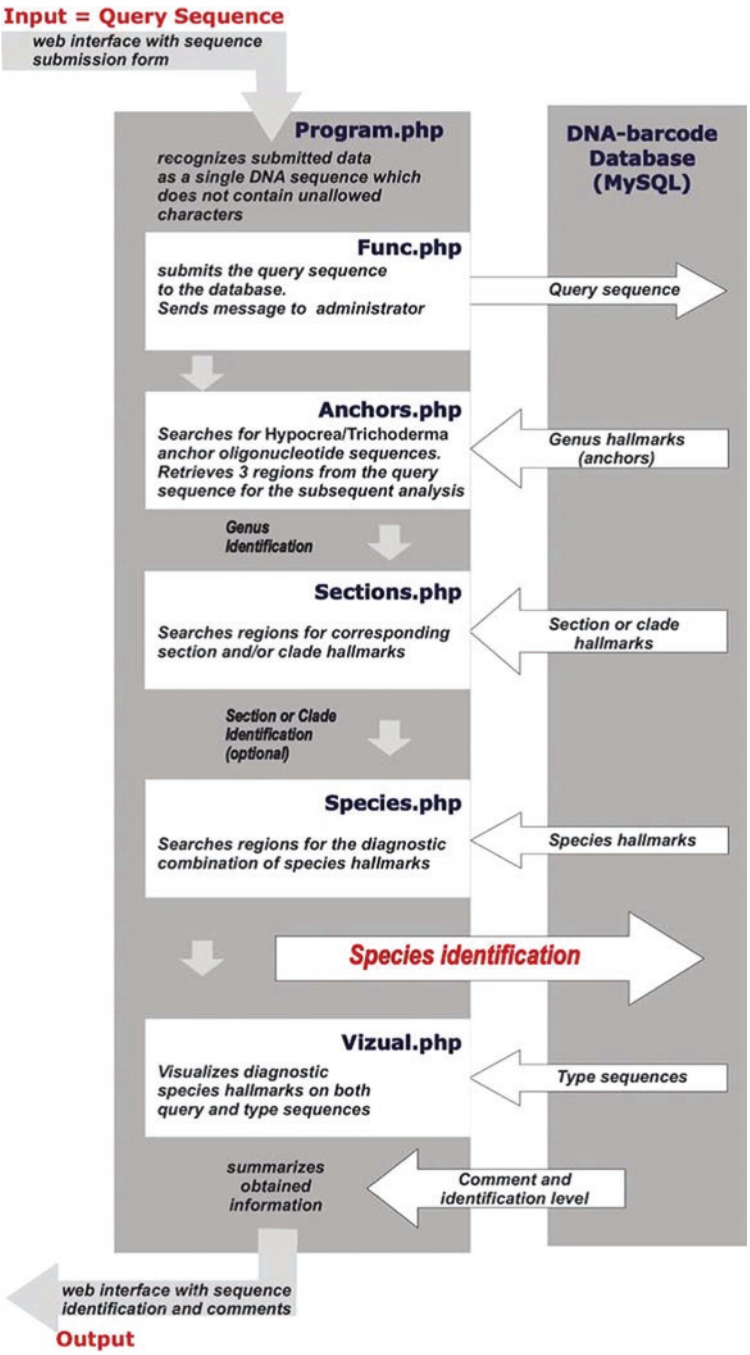

Fig. 5.1 Flowchart of *Trich*OKEY v. 1.0 (Druzhinina et al. 2005)

characters. Otherwise, a species level identification study cannot be corrected or uncorrected and the user has to rely on the person making the misidentification. In this chapter, numbers of protocols or procedures are described for the accurately identifications of *Trichoderma* isolates at the species level.

5.2 DNA Extraction and Purification

DNA extraction method for *Trichoderma* isolates should meet in the following basic criteria: (1) effectiveness extraction, (2) sufficient amount of DNA concentration, (3) discarded any contaminations, (4) high quality and purity of DNA. The purity of the template DNA can be measured by ultraviolet absorbance. The ratio of absorbances at 260 nm and 280 nm (A260/A280) is 1.8, indicating a pure DNA sample. The ratio above 1.8 suggests the sample contaminated by protein or other organic solvent (i.e., phenol). Step-by-step three laboratory protocols of DNA extraction are described in this chapter—one for cetyltrimethyl ammonium bromide (CTAB), second for phenol–chloroform method, and third for commercial DNA extraction kit.

5.3 Obtaining Materials from Which to Extract DNA

5.3.1 Trichoderma Culture

1. Aseptically culture of *Trichoderma* isolates onto PDA agar plate (15 mL PDA with petri dishes).
2. Incubate the cultural agar plates at 28 ± 2 °C (12 h darkness and 12 h light) until 4 days old (Fig. 5.2).

5.3.2 Trichoderma Liquid Culture

1. Suspend 24 g of potato dextrose broth (PDB) (Difco, USA) medium in one litre (1 L) of distilled or deionized water**.**
2. Aliquots of 100 mL medium are transferred into individual 250 mL conical flasks with cotton wool stoppers placed over the flask mouths and then autoclaved at 121 °C, 1.4 kg cm^{-1} for 15 min.
3. After autoclave, keep the flask in laminar flow for cooling.
4. Aseptically remove the cotton wool stoppers from flask mouths and immediately inoculate at least 7 pieces of agar discs of *Trichoderma* cultures (4-day-old actively growing of *Trichoderma* colony cut out by a 5 mm diameter cork borer).

Fig. 5.2 *Trichoderma* isolate is grown onto PDA media for 4 days

Fig. 5.3 *Trichoderma* isolate is grown onto PDB media for 7 day

5. After inoculation of *Trichoderma* isolates, the cotton wool stoppers are again replaced to conical flask and wrapped with aluminum foil, then tight by Parafilm during the growth phase.
6. The inoculated flask is placed static on a bench top at 28 ± 2 °C until day 7 (Fig. 5.3).
7. Harvest the mycelia from the culture(s) by pouring the liquid media containing mycelia growth through a double-layered muslin cloth—alternative: use a non-gauze milk filter or miracloth (CalBiochem cat no. 475855) or several layers of cheesecloth.
8. Allow the culture filtrate to ditch briefly for minute, then wash filtered mycelia using sterilized distilled water, at least twice the filter/flask conduct.

9. Transfer the washed mycelia from the flask with a clean spatula onto a pad of clean paper towels, and blot the mycelia dry or transfer into plastic bags (excess liquid squeezed out) and properly label.
10. Immediately the mycelia can be used or frozen and stored in the deep freezer at −20 °C to −80 °C.
11. The frozen mycelia are ground to make a fine powder using sterile cooled mortar and pestles which are surface-sterilized by swabbing of 99% ethanol just before and after use.
12. Ice cubes are placed surrounding the mortar to maintain the cool temperature. The ground mycelia powder is transferred into microcentrifuge tubes and wrapped with aluminum foil to store at −20 °C to −80 °C for at least several months.

5.4 DNA Extraction Protocols

5.4.1 Phenol–Chloroform Method

1. *Prepare of extraction buffer* (1 M Tris–HCl (pH 8.5), 1 M NaCl (pH 8.5), 1 M EDTA (pH 8.0), 10% (w/v) sodium dodecyl sulfate (SDS) in 1000 mL of double deionized distilled water (ddH_2O)), later should be added the mycelia powder and commercially-prepared phenol buffer (Fluka Biochemika, Switzerland).
2. An approximately 50 mg of ground mycelium powder can take out from the cold storage and place into a sterilized 1.5 mL centrifuge tube.
3. To add 500 μL of the extraction buffer and the mixture homogenized by incubating at 40 °C in a water bath for 8 h.
4. After homogenate, to add 350 μL of phenol buffer followed by 150 μL of 99% (v/v) chloroform and homogenized in an up-down motion in micropipette tips for 10 min.
5. The mixture is centrifuged at 13,000 × *g*/10 min/4 °C.
6. The supernatant layer is transferred to a new 1.5 mL centrifuge tube using a cut-off pipette tip and additionally added a 3 μL of RNAse for reducing or removing any contaminant protein activities.
7. The mixture again incubated at 35–40 °C in water bath for 15 min.
8. After incubation, an equal volume of chloroform (99% v/v) should be added with the mixture of centrifuge tube then inverted at least 10 min for homogenization.
9. Again the mixture is centrifuged at 13,000 × *g*/10 min/4 °C.
10. Again the supernatant layer is transferred into a new 1.5 mL centrifuge tube, then added a 250 μL of iso-propan-2-ol to keep overnight at −20 °C or −80 °C for precipitating the DNA.
11. The mixture is again centrifuged at 13,000 × *g*/4 °C for 10 min to obtain DNA pellets, to observe as clumps of pale white strands.

12. Carefully remove as much liquid by sliding a pipette along the tube, without disturbing the pellets.
13. Following this, the DNA pellet thoroughly wash at least twice with 500 µL of 70% (v/v) ethanol by using an in-out motion of the pipette tips. The ethanol entirely removed from the centrifuge tube
14. After that cover of the tube open for 20 min to air dry the DNA pellets in fume hood.
15. As a final point, the pellets suspended in 50 µL of Tris-EDTA (TE) buffer and measured the absorbance ratio (A260/A280) from 1.8 to 2.0 for observation of high quality pure DNA. The final DNA stocks stored at −20 °C or −80 °C until its use in the next step.

5.4.2 Cetyltrimethylammonium Bromide (CTAB) Method

CTAB buffer solution (100 mL of 1 M Tris–HCl, pH 8.0, 280 mL of 5 M NaCl, 40 mL of 0.5 M EDTA, 20 g of CTAB and 580 mL of sterile distilled water)

1. *Trichoderma* mycelium used for 3 days old grown on agar plate.
2. An approximately 100 mg of mycelia can collect from the edge of *Trichoderma* colony using a sterile inoculating loop and place into 1.5 mL centrifuge tube.
3. A 500 µL of CTAB buffer should add into the samples mixture of centrifuge tube and then homogenized by vortexing (IKA MS1 Minishaker).
4. The mixture incubated at 60 °C for 1 h at 650 rpm (Eppendorf Thermomixer comfort).
5. After that, add 5 µL of ribonuclease A (10 mg mL^{-1}, amresco®) (RNAse A) solution into the sample mixture and incubate again for another 15 min.
6. In fume hood, add a 500 µL of cold phenol–chloroform–isoamylalcohol (25:24:1, v/v) mixture, then inverted the centrifuge tubes for at least 40–50 times and centrifuge at 13,000 rpm for 10 min at 4 °C (Eppendorf Centrifuge 5417R).
7. After centrifugation, the supernatant layer or the upper aqueous layer is transferred into a new centrifuge tube.
8. Add an equal amount of cold isopropanol and mixed by inversion several times.
9. Incubate the mixture tube at −4 °C for 30 min, then centrifuge at 13,000 rpm for 10 min at 4 °C to obtain the DNA pellet.
10. Collect the DNA pellet and wash with 500 µL of 70% cold ethanol at least twice. Each washing step can centrifuge at 13,000 rpm for 10 min at 4 °C.
11. The DNA pellet is drained and dried at 45 °C for 15 min using a speedvac machine (Eppendorf Speed Vac Concentrator Plus 5305).
12. The pellets can dilute in 50 µL of Tris-EDTA (TE) buffer (10 mL of 1 M Tris–HCl (pH 8), 2 mL of 0.5 M EDTA and 988 mL of sterile distilled water).
13. The purity and quantity of the DNA pellet can measure by agarose gel electrophoresis in 1.5–2.0% agarose gel or spectrophotometry method using NanoVue™.
14. The absorbance ratio (A_{260}/A_{280}) from 1.8 to 2.0 is considered as a pure DNA. The final DNA stocks can store at −20 °C until further use.

5.4.3 Procedures of the Commercial DNA Extraction Kit

DNA extraction is conducted using QIAamp DNA extraction kit (Qiagen, Germany) followed by the manufacturer's instruction:

1. Pipetting 20 mL of Qiagen *Proteinase* K and keep in the bottom of 1.5 mL centrifuge tube.
2. Add 200 mL of *Trichoderma* sample to the tube.
3. Add 200 mL of lysis buffer (AL buffer) to the sample. Mix by pulse vortexing for 15 s.
4. Incubate it in a water bath at 56 °C for 10 min.
5. Add 200 mL of ethanol (96–100%) to the sample, mix by gentle pipetting for 15 s.
6. Carefully transfer the mixture from step 5 to the QIAamp mini-spin column (in a 2-mL collecting tube) without wetting the rim. Close the cap, and centrifuged at 8000 rpm for 1 min. Place the QIAamp mini-spin column in a clean 2-mL collecting tube and remove the tube containing filtrate.
7. Carefully open the QIAamp mini-spin column and add 500 mL of buffer AW1 without wetting the rim. Tightly cover the cap and centrifuged at 8000 rpm for 1 min. Place the QIAamp minispin column in a clean 2-mL collecting tube and remove the tube containing filtrate.
8. Again carefully open the QIAamp mini-spin column and add 500 mL of buffer AW2 without wetting the rim. Close the cap and centrifuge at 14,000 rpm for 3 min, followed by an empty spin at 14,000 rpm for 1 min.
9. Place the QIAamp mini-spin column in a new 1.5 mL centrifuge tube and discard the tube containing filtrate. Carefully open the QIAamp mini-spin column and add 200 mL of buffer AE. Incubate at room temperature for 1 min, and then centrifuge at 8000 rpm for 1 min. Discard the column and store the DNA at −20 ° C.

5.5 Polymerase Chain Reaction (PCR) Amplifications

The PCR amplifications protocols are described in below:

5.5.1 Internal Transcribed Spacers (ITS 1, 5.8S, and ITS 2) of the rDNA

1. Two universal primers commonly used: namely, ITS 1 (5′-TCC GTA GGT GAA CCT GCG G-3′ and ITS 4 (5′-TCC TCC GCT TAT TGA TAT GC-3′).
2. Amplify the PCR by in an automated thermocycler (PTC-200 Peltier Thermal Cycler).
3. PCR amplifications can be conducted in 50 μL reaction mixtures containing 5 μL of 10 × PCR buffer, 0.2 μL of 5 Units of *Taq* DNA Polymerase, 1 μL of 10 mM PCR deoxynucleotides triphosphate mix (dNTPs), 3.2 μL of 25 mM

Magnesium Chloride ($MgCl_2$), 1.25 μL of each 10 μM primers (ITS 1 and ITS 4), 34.1 μL of sterilized double distilled water (ddH_2O) and 4 μL of template DNA.
4. All PCR mix reagents can be purchased from FIRST base Laboratories Sdn Bhd, Malaysia.
5. Amplify the ITS regions based on the following procedures: Initial denaturation for 5 min at 95 °C, followed by 35 cycles of denaturation at 94 °C (30 s), annealing temperature at 56 °C or 59 °C (30 s), and extension at 72 °C for 1 min. The amplification can be completed with one additional step of final extension at 72 °C for 10 min.

5.5.2 Translational Elongation Factor 1-α (tef1)

1. Two universal primers commonly used: namely, EF1-728F (5′-CAT CGA GAA GTT CGA GAA GG-3′) and EF1-986R (5′-TAC TTG AAG GAA CCC TTA CC-3′).
2. Amplify the PCR by in an automated thermocycler (PTC-200 Peltier Thermal Cycler).
3. PCR amplification can be conducted in 50 μL reaction mixtures consisted of 5 μL of 10 × PCR buffer, 0.5 μL of 5 Units of *Taq* DNA polymerase, 1.0 μL of 10 mM PCR deoxynucleotides triphosphate mix (dNTPs), 6.0 μL of 25 mM Magnesium Chloride ($MgCl_2$), 1.25 μL of 10 μM each of primers and 10.0 μL of 50 ng of template DNA.
4. PCR amplifications for *tef1* regions can be completed in the following procedures: Initial denaturation for 5 min at 95 °C, followed by 35 cycles of denaturation at 94 °C (30 s), annealing of EF1-728F and EF1-986R primers at 55 °C or 58 °C (30 s), and extension at 72 °C for 1 min. The amplification completed with one additional step of final extension at 72 °C for 10 min.

5.5.3 Calmodulin (cal)

1. Two universal primers used: namely, CAL-228F (5′-GAG TTC AAG GAG GCC TTC TCC C-3′) and CAL-737R (5′-CAT CTT TCT GGC CAT CAT GG-3′).
2. Amplify the PCR by in an automated thermocycler (PTC-200 Peltier Thermal Cycler).
3. PCR amplifications conducted in 50 μL reaction mixtures consisted of 5 μL of 10 × PCR buffer, 0.5 μL of 5 Units of *Taq* DNA polymerase, 1.0 μL of 10 mM PCR deoxynucleotides triphosphate mix (dNTPs), 4.0 μL of 25 mM magnesium chloride ($MgCl_2$), 1.25 μL of 10 μM each of primer and 10.0 μL of 50 ng of template DNA.

4. PCR amplifications for *CAL* regions conducted in the following procedures: Initial denaturation for 5 min at 95 °C, followed by 35 cycles of denaturation at 94 °C (30 s), annealing of CAL-228F and CAL-737R primers at 50 °C (30 s), and extension at 72 °C for 1 min. The amplification completed with one additional step of final extension at 72 °C for 10 min.

5.6 Gel Electrophoresis

5.6.1 Prepare the Gel Apparatus

1. The glass plates are commonly cleaned by polishing with a plastic scouring sponge (non-abrasive) and detergent.
2. The plates should be rinsed with hot water, after that dry with paper towels.
3. Treat the shorter glass plate with gel slick.
4. The gel side surfaces of the plates sterile with 95% ethanol. After that dry them with a paper towel and then assemble the plate, spacers, and boot according to the manufactures instructions (Fig. 5.4).

5.6.2 Visualisation of DNA

1. An approximately 4 μL of each PCR products or template DNA could be electrophoresed in 1.5–2.0% of agarose gel using 1× Tris-borate-EDTA (TBE) running buffer (89 mM Tris-base, 89 mM boric acid and 2 mM EDTA, pH 8) at 100 Volt for 45 min. During electrophoresis, an electrical field is applied so that the negatively charged DNA fragments move toward the positive electrode. The DNA fragment moved through the agarose medium has inversely proportional to its molecular weight.
2. To estimate an approximate sizes of the amplified regions by referring to the 50 or 100 bp DNA ladder.
3. The gel can stain in Ethidium bromide (EtBr) for 15 min then visualize under UV-light and photographed using Alphalmager® HP (Alpha Innotech) (Fig. 5.5).

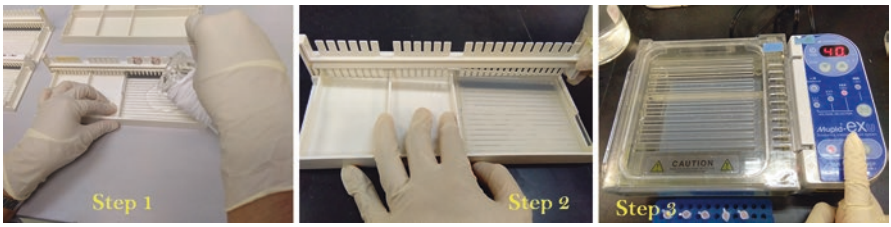

Fig. 5.4 The preparation procedure of gel electrophoresis

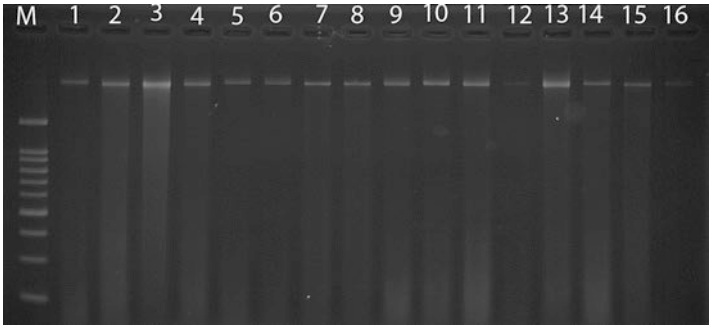

Fig. 5.5 Agarose gel electrophoresis of genomic DNA extracted from *Trichoderma* isolates. The *white error* indicates the genomic DNA extracted. Lane M: 100 bp DNA ladder, lines 1–15: *Trichoderma* isolates, and Line 16, negative control

5.7 Purification and Sequencing of PCR Products

1. PCR products of each isolates can purify using QIAquick® PCR Purification Kit (QIAGEN, Chatsworth, CA, USA).
2. Buffer PB (Binding Buffer) can add to PCR products in a 5:1 portion (Buffer PB: PCR product). The mixture is turned to yellow in color.
3. To bind DNA, the mixture is transferred to a QIAquick column which is placed in a 2 mL collection tube and centrifuged for 1 min at 13,000 rpm.
4. The flow-through is cast off and the QIAquick column is placed back into the same collection tube.
5. Then, 750 μL of buffer PE (Washing Buffer) is added to the QIAquick column and centrifuged for 1 min at 13,000 rpm.
6. The flow-through again cast-off and the QIAquick column is placed back into the collection tube and centrifuged for additional 1 min to remove the remaining washing buffer.
7. Then, the QIAquick column is placed into sterile 1.5 mL centrifuge tube and added 30 μL of buffer EB (Elution Buffer) to the centre of the QIAquick membrane.
8. The tube can stand for 5 min, then centrifuged for 1 min at 13,000 rpm to elute the purified PCR products.
9. The purified PCR products are completed to gel electrophoresis in 1.5% of agarose gel using 1× TBE buffer and its concentration is determined by using Nanovue machine (Fig. 5.6).
10. The purified PCR products (~15 μL) are directly sent for sequencing at First BASE Laboratories Sdn Bhd or other sequencing laboratory.

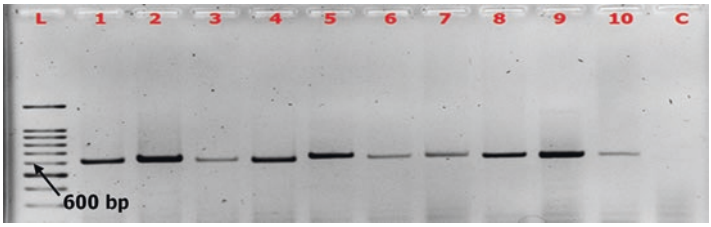

Fig. 5.6 A representative picture of the agarose gel showing the PCR purification products of the ITS 1 and ITS 2 regions of all *Trichoderma* isolates with the expected amplicon size (600 bp). Lane 1: 100 bp of DNA Ladder, lanes 1–10: *Trichoderma* isolates, Lane C: Negative control

5.8 Sequence Analysis and Phylogenetic Inference of the ITS 1 and ITS 2 Regions of the rDNA, Translational Elongation Factor 1-α (*tef1*), and Calmodulin (*cal*) Genes

1. The DNA sequencing results of the ITS1 and ITS2 regions of the rDNA, *tef1* and *cal* genes are analyzed using Bioedit Sequence Alignment Editor (BioEdit).
2. The sequences are checked for quality, trimmed, and manually edited.
3. The forward and reverse sequences are assembled and an agreement sequence is produced from each alignment made.
4. The DNA sequences are aligned by CLUSTAL-W algorithm. The Clustal W program is set to default parameters. The default parameters of multiple alignment in Clustal W is consisted of gap penalty, gap length penalty, delay divergent seqs (%) and DNA translation weight, are set as respectively 10.00, 0.20, 30 and 0.50.
5. DNA sequencing (ITS, *tef1* and *cal)* are compared to those in existing databases using the NCBI BLAST and *Trich*OKEY v 2 program, which are available online from the National Centre for Biotechnology Information, Basic Local Alignment Search Tool (BLAST) (http://blast.ncbi.nlm.nih.gov) and the International Subcommission on *Trichoderma* and *Hypocrea* Taxonomy (ICTF) (ISTH, www.isth.info). Summary of the five highest subject species from BLAST result can obtain.
6. The phylogenetic trees of ITS, *tef1* and *cal* genes sequences are constructed using Molecular Evolutionary Genetic Analysis 6 (MEGA 6) software package or others software available online database.

5.9 Identification of *Trichoderma* Species by Sequencing

DNA sequencing is a quick molecular based classification of *Trichoderma* isolates. The genus and species levels are accurately identified using an oligonucleotide DNA barcode; it combination of several oligonucleotides specifically allocated within the internal transcribed spacers 1 and 2 (ITS1 and 2) regions of the rDNA

gene, translational elongation factor 1-α (*tef1*) and calmodulin (*Cal*) sequences. In this chapter is included proper description about 12 *Trichoderma* species in below as referred to mycologist/scientists/researchers/lab technicians for the correctly identification mentioned species.

5.9.1 Trichoderma asperellum

ITS1 and 2 sequence(s) in FASTA or text format.

cgttaccaaactgttgcctcggcggggtcacgccccgggtgcgtcgcagccccggaaccaggcgcccgc
cggaggaaccaaccaaactctttctgtagtcccctcgcggacgtatttctttacagctctgagcaaaaattcaa
aatgaatcaaaactttcaacaacggatctcttggttctggcatcgatgaagaacgcagcgaaatgcgataagt
aatgtgaattgcagaattcagtgaatcatcgaatctttgaacgcacattgcgcccgccagtattctggcgggca
tgcctgtccgagcgtcatttcaaccctcgaacccctccgggggatcggcgttggggatcgggacccctcac
acgggcgccggcccctaaatacagtggcggtctcgccgcagcctctcctgcgcagtagtttgcacaactcg
caccgggagcgcggcgcgtccacgtccgtaaaacaccca

Or

gcgcccgccggaggaaccaaccaaactctttctgtagtcccctcgcggacgtatttctttacagctct-
gagcaaaaattcaaaatgaatcaaaactttcaacaacggatctcttggttctggcatcgatgaagaacg-
cagcgaaatgcgataagtaatgtgaattgcagaattcagtgaatcatcgaatctttgaacgcacattgcgccc-
gccagtattctggcgggcatgcctgtccgagcgtcatttcaaccctcgaacccctccgggggatcggc-
gttggggatcgggacccctcacacgggcgccggcccctaaatacagtggcggtctcgccg-
cagcctctcctgcgcagtagtttgcacaactcgcaccgggagcgcg

Translational elongation factor 1-α (*tef1*) sequence(s) in FASTA or text format.

attatatgcccgacaattctgttctcagttttgtctttctttttttcagcatcaccccgctttgccagcctacctacccc
tcctttggcacagcaaaaaattttctcgctgccttgtttggcttttagtggggtgtcaattttgtttgacggcaacc
ccactatcgccactgtacctctttccatcatccaccacatgcttttgttcaatcgcatcgtctattttcaatatctctt
gttcattatgctgatcatgc

Or (Holmes et al. 2004)

atcgagaagttcgagaaggtaagctcatttcactgcttttcccatcaatttttggcacaatcatatgcccga-
caattctgctctcagtttttgtctttttttttccagcgtcaccccgctttgccagtctacctacccctcctttggca-
cagcaaaaattttctggctgccttgtttggcttttagtggggtgtcaaatttttttggcagcaaccccgctatcgc-
cactgcacctcttccatcacccaccacatgctatttgctcaatcgcgtcgtctttttttgttcattatgctgatcat-
gcttcaatcaataggaagccgccgaactcggcaagggttccttcaagtatgcgtgggttcttgacaagct-
caaggccgagcgtgagcgtggtatcaccatcgacattgccctctggaagttcgagactcccaagtactatgt-
caccgtcattggtatgttttggactcttctctctagctatcgacattccaagtccgccattctaacat-
gctcttcccacagacgctcccggtcaccgtgatttcatcaag

Calmodulin (*cal*) sequence(s) in FASTA or text format.

acataaaaagatngtaatttgggatgaactgcaggcgatttgggcactaacccggtggctacaggacaagg
acggcgatggtacgtagtggctagtgacgcgatactcctctttcccctcctcaccgtcccccttattttgtgcgg
gctactactagagcgcctgaacctgcgatagcgatcgaaatttccccgactggccatttttatgacggatagg
aggacgcaaagctaacagccttgacttcgcaggccagatcaccaccaaggagctgggcactgtcatgcgct
ccctgggccagaaccccctccgagtcagagctgcaggacatgatcaacgaggttgatgccgacaacaatgg
atccatcgatttccctggtatgtcaatagcagaaacgcatggacggctaccgaatacggactaatctagagcg
gtgaagagttccttaccatgatggccagaaagatga

Or

caggcgatttgggcactaacccggtggctacaggacaaggacggcgatggtacgtagtggctagt-
gacgcgatactcctctttcccctcctcaccgtcccccttattttgtgcgggctactactagagcgcctgaacct-
gcgatagcgatcgaaatttccccgactggccatttttatgacggataggaggacgcaaagctaacagcctt-
gacttcgcaggccagatcaccaccaaggagctgggcactgtcatgcgctccctgggccagaaccccctcc-
gagtcagagctgcaggacatgatcaacgaggttgatgccgacaacaatggatccatcgatttccctggtat-
gtcaatagcagaaacgcatggacggctaccgaatacggactaatctagagcggtgaagagttccttacc

5.9.2 Trichoderma harzianum

ITS1 and 2 sequence(s) in FASTA or text format.

aagggccctcgggactcccgcttttggttacccctcggatccgggtgtttccccccctgtggacgcgccgcgc
tcccgatgcgagtgtgcaaactactgcgcaggagaggctgcggcgagaccgccactgtatttcggagacg-
gccacccgctaagggagggccgatccccaacgccgacccccccggaggggttcgagggttgaaatgacgc
tcggacaggcatgcccgccagaatactggcgggcgcaatgtgcgttcaaagattcgatgattcactgaattct
gcaattcacattacttatcgcatttcgctgcgttcttcatcgatgccagaaccaagagatccgttgttgaaagtttt
gattcattttcgaaacgcctacgagaggcgccgagaaggctcagattataaaaaaaaaaacccgcgagggg
gtatacaataagagttttggttggtcctccggcgggcgccttggtccggggctgcgacgcacccggggcag
agatcccgccgaggcaacagtttggtaacgttcacattgggtttgggagttgtaaactcggtaatgatccctcc
gcaggttcacctacggaagca

Or

gggccctcgggactctcgcttattgattgccccacggttccctccgaatcccccccgtttttgcattgcccc
agggttgccagtgtacaccctcctgtttaggagaggctgcggcgagaccgccactgtctttcggatacggcc
acccgctaagggagggccgatccccaacgccgacccccccggaggggttcgagggttgaaatgacgctcg
gacaggcatgcccgccagaatactggcgggcgcaatgtgcgttcaaagattcgatgattcactgaattctgc
aattcacattacttatcgcatttcgctgcgttcttcatcgatgccagaaccaagagatccgttgttgaaagttttga
ttcattttcgaaacgcctacgagaggcgccgagaaggctcagattataaaaaaaaaaaacccgcgaggggt
atacaataagagttttggttggtcctccggcgggcgccttggtccggggctgcgacgcacccggggcagag
atcccgccgaggcaacagtttggtaacgttcacattgggtttgggagttgtaaactcggtaatgatccctccgc
aggttcacctacggaa

Translational elongation factor 1-α (*tef1*) sequence(s) in FASTA or text format.

Attcggacggtttgagtgctaacccagcttgctacaggacaaggacggtgatggtacgtagca-
gcgagtgacgcgacaccacccctctccccctttgcgaataggcaccggagcaccgaatgacttgctgccg
accgatatcttccttgccagccccttttcatgagatctaagcaaagaaaagaactgacaggcttgaccccgca
ggccagatcaccaccaaggagctgggcaccgtgatgcgctccctcggccagaatccctccgaatcagagc
tgcaggacatgatcaacgaggtcgatgccgacaacaacggatctatcgatttccccggtatgtcaaaagtc
aaagtgttgggtcgttg

Or (Chaverri et al. 2001)

tcgtacgtacaatcctgttccctcacgtcggcatcattcgccggtctgattctcaaacacttgtgctaac-
catcgccttctaggggtgcgtattccatcaatcatcttgaatgagatcgatcgaacacaatactgacttgctata-
acagccacgtcgactccggaaagtcgaccaccgtaagttgcaccctctcttgctccgatatcaaacgtcgttt-
gatgggggacacctactctagagcacagggctaaccattcatcatacagaccggtcacttgatctaccagt-
gcggtggtatcgaccgtcgtaccatcgagaagttcgagaaggtaagcttcaactgatttttcgcctc-
gattctttcctccttcacattcaattgtgcccgacaattctgcagagaattttcgtgtcgacaatttttcat-
caccccgctttccgttacccctcctttgcagcgacgcaaaatttttttgctgtcgtctggtttt-
tagtggggttctctgtgcaaccccactagctcactgctttttcctgcttcactctcacttcctcgtcatcattcaa-
cacg

Calmodulin (*cal*) sequence(s) in FASTA or text format.

attcggacggtttgagtgctaacccagcttgctacaggacaaggacggtgatggtacgtagcagcgagtga
cgcgacaccacccctctccccctttgcgaataggcaccggagcaccgaatgacttgctgccgaccgatatc
ttccttgccagccccttttcatgagatctaagcaaagaaaagaactgacaggcttgaccccgcaggccagatc
accaccaaggagctgggcaccgtgatcgctccctcggccagaatccctccgaatcagagctgcaggacat
gatcaacgaggtcgatgccgacaacaacggatctatcgatttccccggtatgtcaaaagtcaaagtgttgggt
cgttg

5.9.3 Trichoderma hamatum

ITS1 and 2 sequence(s) in FASTA or text format.

Ctcggcggggtcacgccccgggtgcgtaaaagccccggaaccaggcgcccgccggaggaaccaacc
aaactctttctgtagtcccctcgcggacgtatttcttacagctctgagcaaaaattcaaaatgaatcaaaactttc
aacaacggatctcttggttctggcatcgatgaagaacgcagcgaaatgcgataagtaatgtgaattgcagaat
tcagtgaatcatcgaatctttgaacgcacattgcgcccgccagtattctggcgggcatgcctgtccgagcgtc
atttcaaccctcgaacccctccgggggatcggcgttggggatcgggacccctcaccgggtgccggccctg
aaatacagtggcggtctcgccgcagcctctcctgcgcagtagtttgcacaactcgcaccgggagcgcggc
gcg

Or

tgtgaacgttaccaaactgttgcctcggcggggtcacgccccgggtgcgtaaaagccccggaaccag-
gcgcccgccggaggaaccaaccaaactctttctgtagtcccctcgcggacgtatttcttacagctctgag-
caaaaattcaaaatgaatcaaaactttcaacaacggatctcttggttctggcatcgatgaagaacgcagc-
gaaatgcgataagtaatgtgaattgcagaattcagtgaatcatcgaatctttgaacgcacattgcgcccgc-
cagtattctggcgggcatgcctgtccgagcgtcatttcaaccctcgaacccctccgggggatcggc-
gttggggatcgggacccctcaccgggtgccggccctgaaatacagtggcggtctcgccgcagcctctcct-
gcgcagtagtttgcacaactcgcaccgggagcgcggcgcgtccacgtccgtaaaacaccc

Translational elongation factor 1-α (*tef1*) sequence(s) in FASTA or text format (Haas et al. 2016)**.**

cgtaagttgcaatcccattcttctcttcagcggcattctctgctgctggctatgaatgacatcgtgctaaccgtca
tcttctaggggttcgtatttctccatcaaactgctttcgatcgactccaatactgacttgctacaacagccacgtc
gactccggcaagtctaccaccgtgagtaatatccaattcctcaagcctctctgccatcggctctgtcggtcggc
gcggggtatatcaagtatgaacgcaccaagctaacgttttccaatagactggtcacttgatctaccagtgcggt
ggtattgacaagcgtaccatcgagaagttcgagaaggtaagttcatttcgctgctttttattccttttgggcaca
attgtgccagacaattctgttctcagtcttgtcaacatttttttcccaccaagcatcgcaccccgctttgtctgccta
cctacccctcctttggcacagcaaaaattttctggctgccttggttggtttttagtggggtgccaaatttttggcag
tgaccccgccatcgccactgttcctcatgcactacccaacacatgctacgtatcaactgcttggttcattgtgct
aatcatacttcaatcaataggaagccgccgaactcggcaagggttccttcaagtatgcgtgggttcttgacaa
gctcaaggccgagcgtgagcgtggtatcaccatcgacattgccctgtggaagttcgagactcccaagtacta
tgtcaccgtcattggtatgttttcagtccgactggtcactatcccaacatcatcatgctaacgtgcga

Calmodulin (*cal*) sequence(s) in FASTA or text format (Samuels et al. 2006).

tgagtctctagacagtcttttgcgacaaatctgctggcgatttgggtactaatctgctggctacaggacaagga
cggcgatggtacgtagtacctagtgacgcgatactcctcttttcccctcctcaccgtcccccctattttgtgcgag
ctatcactagagcgcctgaacctgcgataccgatcgaaatatccccggctggcaatttttatgacgaataaac
ggacaagaaactaacagacttgacttggcaggccagatcaccaccaaggagctgggcactgtcatgcgctc
tttgggccagaaccccctccgagtcagagctgcaggacatgatcaacgaggttgatgccgacaacaacggat
ccatcgatttccctggtatgtcaatagcagaaacacatggacggctgccggatacgggctaatctagagcgg
tgaagagttccttacc

5.9.4 Trichoderma reesei

ITS1 and 2 sequence(s) in FASTA or text format.

ctcggcgggattctctgccccgggcgcgtcgcagccccggatcccatggcgcccgccggaggaccaact
caaactctttttttctctccgtcgcggcttccgtcgcggctctgttttacctttgctctgagcctttctcggcgaccct
agcgggcgtctcgaaaatgaatcaaaactttcaacaacggatctcttggttctggcatcgatgaagaacgcag
cgaaatgcgataagtaatgtgaattgcagaattcagtgaatcatcgaatctttgaacgcacattgcgcccgcca
gtattctggcgggcatgcctgtccgagcgtcatttcaaccctcgaacccctccgggggggtcggcgttgggga
tcggcccctcaccgggccgcccccgaaatacagtggcggtctcgccgcagcctctcctgcgcagtagtttg
cacactcgcaccgggagcgcggcgcggccacagc

Translational elongation factor 1-α (*tef1*) sequence(s) in FASTA or text format.

gcatctggcgaacgaatgctgtgccgacacgatttttttttttcatcaccccgctttctcctacccctccttcgagc
gacgcaaatttttttgctgccttacgagttttagtggggtcgcacctcacaaccccactactgctctctggccg
ctccccagtcacccaacgtcatcaacgcagca

or (Nakari et al. 1993)

ctgactctgcttgcctcgcagcgatctagtcaagaacaacacagctctcacgctacatcacacaaacc-
gtcaaaatgggtaaggaggacaagactcacatcaacgtggtcgtcatcgtacgtattttccgatccctcatcg-
gcgtcatctgccagtctgattccaagaatcaccgtgctaaccatataccatctaggggtgcgtattccatcaat-
catcttgagccagatcgaccgaacatacgatactgactttgctacgacagccacgtcgactccggcaagtc-
taccaccgtgagtaaacacccattccactccacgaccgcaagctccatcttgcgcgtggcgtctctgcgat-
gaacatccgaaactgacgttctgttacagactggtcacttgatctaccagtgcggtggtatcgacaagcgtac-
cattgagaagttcgagaaggtaagcttcgttccttaaatctccagacgcgagcccaatctttgcccatct-
gcccagcatctggcgaacgaatgctgtgccgacacgatttttttttttcatcaccccc-
gctttctcctacccctccttcgagcgacgcaaatttttttttgctgccttacgagttttagtggggtcgcacctca-
caaccccactactgctctctggccgctccccagtcacccaacgtcatcaacgcagcagttttcaatcagc-
gatgctaaccatattccctcgaacaggaagccgccgaactcggcaagggttccttcaagtacgc-

(continued)

(continued)

gtgggttcttgacaagctcaaggccgagcgtgagcgtggtatcaccatcgacattgccctctggaagttc-
gagactcccaagtactatgtcaccgtcattggtatgttggcagccatcacctcactgcgtcgttgacacat-
caaactaacaatgccctcacagacgctcccggccaccgtgacttcatcaagaacatgatcactgg-
tacttcccaggccgactgcgctatcctcatcatcgctgccggtactggtgagttcgaggctggtatctc-
caaggatggccagacccgtgagcacgctctgctcgcctacaccctgggtgtcaagcagctcatcgtcgc-
catcaacaagatggacactgccaactgggccgaggctcgttaccaggaaatcatcaaggagacttc-
caacttcatcaagaaggtcggcttcaaccccaaggccgttgctttcgtccccatctccggcttcaacggt-
gacaacatgctcacccccctccaccaactgcccctggtacaagggctgggagaaggagaccaaggctg-
gcaagttcaccggcaagaccctccttgaggccatcgactccatcgagccccccaagcgtcccacgga-
caagcccctgcgtcttcccctccaggacgtctacaagatcggtggtatcggaacagttcccgtcggccg-
tatcgagactggtgtcctcaagcccggtatggtcgttaccttcgctccctccaacgtcaccactgaagt-
caagtccgtcgagatgcaccacgagcagctcgctgagggccagcctggtgacaacgttggtttcaacgt-
gaagaacgtttccgtcaaggaaatccgccgtggcaacgttgccggtgactccaagaac-
gacccccccatgggcgccgcttctttcaccgcccaggtcatcgtcatgaaccaccccggccaggtcggt-
gccggctacgcccccgtcctcgactgccacactgcccacattgcctgcaagttcgccgagctcctcgagaa-
gatcgaccgccgtaccggtaaggctaccgagtctgcccccaagttcatcaagtctggtgactccgccatc-
gtcaagatgatcccctccaagcccatgtgcgttgaggctttcaccgactaccctcccctgggtcgtttcgcc-
gtccgtgacatgcgccagaccgtcgctgtcggtgtcatcaaggccgtcgagaagtcctctgccgccgcc-
gccaaggtcaccaagtccgctgccaaggccgccaagaaataagcgatacccatcatcaacacctgat-
gttctggggtccctcgtgaggtttctccaggtgggcaccaccatgcgctcacttctacgacgaaacgat-
caatgttgctatgcatgagactcgactatgaatcgaggcacggttaattgagaggctgggaataagggttc-
catcagaacttctctgggaatgcaaaacaaaagggaacaaaaaaactagatagaagtgaattcatgacttc-
gacaa

Calmodulin (*cal*) sequence(s) in FASTA or text format.

cctttactgcggcaacccggtagggggttgttttcagggtgctgaccgagctgctctacaggacaaggacgg
cgatggtacgtgatggcgagtgacgcgacaacacacctattgccctctcgacaaagccgcaccgaagcact
ttgtgccgatcgatcactctatcgtcgactcgaatcatgatacatggacaagaaactgacaggcttgacctc

Or (Druzhinina et al. 2012)

tcaaaagggaggccttctccctctttgtgagttttttcaacgtcctttactgcggcaacccggtaggggttg-
gttttcagggtgctgaccgagctgctctacaggacaaggacggcgatggtacgtgatggcgagtgacgc-
gacaacacacctattgccctctcgacgaagccgcaccgaagcactttgtgccgatcgatcactctatcgt-
caactcgaatcatgatacatggacaagaaactgacaggcttgacctcgtaggccagatcaccac-
caaggagttgggcaccgtgatgcgctctctcggccagaacccttccgagtcggagctgcaggacatgat-
caacgaggttgacgccgacaacaacggctccatcgacttccctggtatgtgaattgttgggagatttggtg-
gttgaggtatacgggctgacgtgga gcggtgaagaatttctcaccatgatggcacgaaaaatg

5.9.5 *Trichoderma longibrachiatum*

ITS1 and 2 sequence(s) in FASTA or text format.

acgttaccaatctgttgcctcggcgggattctcttgccccgggcgcgtcgcagccccggatcccatggcgcc
cgccggaggaccaactccaaactctttttttctctccgtcgcggctcccgtcgcggctctgttttatttttgctctg
agcctttctcggcgaccctagcgggcgtctcgaaaatgaatcaaaactttcaacaacggatctcttggttctgg
catcgatgaagaacgcagcgaaatgcgataagtaatgtgaattgcagaattcagtgaatcatcgaatctttga
acgcacattgcgcccgccagtattctggcgggcatgcctgtccgagcgtcatttcaaccctcgaaccccctcc
gggggggtcggcgttggggatcggcccctcaccgggccgcccccgaaatacagtggcggtctcgccgca
gcctctcctgcgcagtagtttgcacactcgcaccgggagcgcggcgcggccacagccgtaaaacaccccaaa
ctt

Translational elongation factor 1-α (*tef1*) sequence(s) in FASTA or text format (Druzhinina et al. 2005).

ttcacatcaacgtggtcgttatcgtacgtactttcccctcccgtcggcctcatctgccggtctgatttcaaggaac
attgtgctaaccattgtctaggggtgcgtattccatcaaaaatcatcttgaaacagatcggccgaacacaaaat
actgactttgctgcgacagccacgtcgactccggcaagtctaccaccgtgagtaaacaccgagctttcagtc
cacgtcaagctacggcctgcgcgtggcgtctctgcgatgaacatgccagctaacgttgccttgcagactggt
cacttgatctaccagtgcggtggtatcgacaagcgtaccattgagaagttcgagaaggtaagcttgagatccc
ttcaattttcggacgatttcctgtgcctctgcccaacatctttttttttcaccaccccgctttctcctacccctcctttg
ggcgacgcaaatttttttttgttgcgtttcgggttttagtggggatgcacctccagcaaaccactatcctctgccg
ccctctgctctcgtctccaacacctttggcgcttgcgtcatcaaccttccaacagtctgcgcagcaatgctaatc
attttcccctcaacaggaagccgccgaactcggcaagggttccttcaagtacgcgtgggttcttgacaagctc
aaggccgagcgtgagcgtggtatcaccatcgacattgccctctggaagttcgagactcccaagtactatgtc
accgtcattggtatgtttgatcccgtgcactcattgcatcatcgccacaacaacatactaatgccctctgacaga
cgtcccggccaccgtgatt

Calmodulin (*cal*) sequence(s) in FASTA or text format (Druzhinina et al. 2008).

tgcatactctgctgcggcaaacctggtagggaaggcgttttcagggtgctaaccgagctgctacaggacaag
gacggtgatggtacgtgacggcgagtgacgcgacaacacacctatttctctctctacgagcccgcaccgaa
gcattcattgccgatcgatagccctctcgcacgctcgaattattgataaatggacacgataaactgacaggctt
gaccccgtaggccagatcaccaccaaggagctgggcaccgtcatgcgctctctcggccagaaccccttccg
agtcggagctgcaggacatgatcaacgaggttgacgccgacaacaacggttccatcgacttccctggtacgt
gacttgtcgcaagatttggtggccaaggtatatgggctaacatggagcggtgaagaatttctcaccatgatgg
ccagaaagatgagccccacc

5.9.6 Trichoderma spirale

ITS1 and 2 sequence(s) in FASTA or text format.

cgggatctctgccccgggtgcgtcgcagccccggaccaaggcgcccgccggaggaccaaccaaaactct
tttgtatacccccctcgcgggtttttatatatctgagccatctcggcgcctctcgtaggcgtttcgaaaatgaatca
aaactttcaacaacggatctcttggttctggcatcgatgaagaacgcagcgaaatgcgataagtaatgtgaatt
gcagaattcagtgaatcatcgaatctttgaacgcacattgcgcccgccagtattctggcgggcatgcctgtcc
gagcgtcatttcaaccctcgaacccctccgggggggtcggcgttggggatcggccctttacggggccggccc
cgaaatacagtggcggtctcgccgcagcctctcctgcgcagtagtttgcacactcgcatcgggagcgcggc
gcgtccat

Or

acgttaccaaactgttgcctcggcgggatctctgccccgggtgcgtcgcagccccggaccaaggc-
gcccgccggaggaccaaccaaaactcttttgtatacccccctcgcgggtttttatatctgagccatctcggc-
gcctctcgtaggcgtttcgaaaatgaatcaaaactttcaacaacggatctcttggttctggcatcgat-
gaagaacgcagcgaaatgcgataagtaatgtgaattgcagaattcagtgaatcatcgaatctttgaacgca-
cattgcgcccgccagtattctggcgggcatgcctgtccgagcgtcatttcaaccctcgaacccctcc-
gggggggtcggcgttggggatcggcccttttacggggccggccccgaaatacagtggcggtctcgccg-
cagcctctcctgcgcagtagtttgcacactcgcatcgggagcgcggcgcgtccattgccgtaaaa-
cacccaactt

Translational elongation factor 1-α (*tef1*) sequence(s) in FASTA or text format (Samuels 2006).

gagaaggtaagctcaatcaactgattctcgcctcaatttccccttcacattcaattgtgctcgacaattctgcacg
gaattctcttgtcaacaatttttcaccaccccgctttcgcttccattacccctcctttgcagcgacgcaaatttttttg
cagctctaggttttagtggggtgcaccagcaaccccaccgccgcctatcgctgctttttgcccttcactacgac
tacacagttactcattttcaacgatgctaaccatctttccctcaacaggaagccgccgaactcggcaagggttc
cttcaagtacgcttgggttcttgacaagctcaaggccgagcgtgagcgtggtatcaccattgatatcgctctgt
ggaagttcgagactcccaagtactatgtcaccgtcattggtatgtctcatttattacctccatgctacaattgcaa
ctcggtgctaatgcaaacattacagacgccccccggccaccgtgatttcatcaagaacatgatcactggtactt
cccaggccgattgcgccattctcatcattgccgccggtactggtgagttcgaggctg

Calmodulin (*cal*) sequence(s) in FASTA or text format (Chaverri et al. 2008).

ccttctccctctttgtgagtctttaactcgtttcttgtggcaattcgagcggtttgagtgctaacccagctgctaca
ggacaaggacggtgatggtacgtaactgcgagtgacgcgacaccactcttccccctttgcgaacaggca
ccggagcaccgaatgatttgctgccgaccgagattttcttgcaggcctgttttatgatatttgggtaaagaaaaa
aaactgacaggcttgaccccgcaggccagatcaccaccaaggagctgggcaccgtgatgcgctctctggg
ccagaaccctccgagtcagagctgcaggacatgatcaacgaggttgatgccgacaacaacggatctatcg
atttccctggtatgtcaatagtggaaatatttggtcgttagcgcatttcatctaatacgaagcggtgtagaattcct
gaccatgat

5.9.7 Trichoderma brevicompactum

ITS1 and 2 sequence(s) in FASTA or text format.

gaacgttaccaaacatgttgcctcggcgggatttctgccccgggcgcgtcgcagccccggaccaaggcgcccg
ccggaggaccaatttacaaactcttttgatatcccatcgcggattctttacattctgagctttctcggcgctcctagcg
agcgtttcgaaaatgaatcaaaactttcaaaactttcaacaacggatctcttggttctggcatcgatgaagaacgca
gcgaaaatgcgataagtaatgtgaattgcagaattcagtgaatcatcgaatctttgaacgcacattgcgcccgcca
gtattctggcgggcatgcctgtccgagcgtcatttcaaccctcgaacccctccgggggggtcggcgttggggatcg
gcacntacctgccggccccgaaatacagtggcggtctcgccgcagcctcncctgcgcagtagtttgcacacnc
gcaccgggagcgcggcgcgtccacggccgtaaaacaacccaa

Translational elongation factor 1-α (*tef1*) sequence(s) in FASTA or text format (Kraus et al. 2004).

atgcaattgtgcccgacaattctcagttatctgaggattttgtgtcaatttttttttcttcgtggtcaccccgctttcac
tgcctacccctcctttggtacagacctgcaaatttttttgctgccttactaggttttagtggg

Calmodulin (*cal*) sequence(s) in FASTA or text format (Chaverri and Samuels 2013).

acggtttgggtgctaacccagctcctacaggacaaggacggtgatggtacgtagcagcgagtgacgcgac
accacccctctccccctttgcgaacaggcaccggagcaccgaatgacttgcttccgaccgatatttgcaacc
ccctttcatgatatctgagcaaagaaacgaactgacaggcttgaccccgcaggccagatcaccaccaagga
gctgggcaccgtgatgcgctccctcggccagaaccctccgagtcagagctgcaggacatgatcaacgag
gtcgatgccgacaacaacggatctatcgatttccccggtatgtcaaaagtcaaagtgttgggccgttgccaca
ttcgagctaatacggagcggtgtagaattcctgaccatgatgg

5.9.8 Trichoderma erinaceum

ITS1 and 2 sequence(s) in FASTA or text format.

agtcccctcgcggacgttatttcttacagctctgagcaaaaaattcaaaatgaatcaaaactttcaacaacggat
ctcttggttctggcatcgatgaagaacgcagcgaaatgcgataagtaatgtgaattgcagaattcagtgaatca
tcgaatctttgaacgcacattgcgcccgccatattctggcgggcatgcctgtccgagcgtcatttcaaccctcg
aacccctccgggggggtcggcgt

Or

aaactgttgcctcggcggggtcacgccccgggtgcgtcgcagccccggaaccaggcgcccgccg-
gagggaccaaccaaactctttactgtagtcccctcgcggacgttatttcttacagctctgag-
caaaaaattcaaaatgaatcaaaactttcaacaacggatctcttggttctggcatcgatgaagaaggcagc-
gaaatgcgataagtaatgtgaattgcagaattcagtgaatcatcgaatctttgaacgcacattgcgcccgc-
cagtattctggcgggcatgcctgtccgagcgtcatttcaaccctcgaacccctccgggggggtcggc-
gttggggatcgggaaccccctcagacgggatcccggccccgaaatacagtggcggtctcgccg-
cagcctctcctgcgcagtagtttgcacaactcgcaccgggagcgcggcgcgtcc

Translational elongation factor 1-α (*tef1*) sequence(s) in FASTA or text format (Hoyos-Carvajal et al. 2009).

ttgcatttctcattcctctcgtcagcggcactctctctgccgttgacactgatgcgcataacattttgctgaccata
gtcgtctaggggttcgtatttctccatatagctttcgatcgactccaatactgacttgctacaacagccacgtcga
ctccggcaagtctaccaccgtgagtaacctccaattcctcgagccctatcgccatcgactctgtcggtcggcg
cggggtatcttcaacatgagcgcatccagctgacattttcccaatagactggtcacttgatctaccagtgcggt
ggtattgacaagcgtaccatcgagaagttcgagaaggtaagctattttcacagcttttctctatgcattcatagc
acaatcgtgtcgacaattcttgtcctcagacttgtcgatttttcctcacggtgtcacaccccgctttacctgcccta
cccctcctttggctcagcaaaaaaattttctggttgccttgtttggtttgtagtggggtgccaacttttggcagca
accccgctatcgccactgtccttcatccatcatcccaatgccttgttctcactcaatcgcatcgtcttttgcctcaa
tttatgattcattgtgctaatcatgtttcaaccaataggaagccgccgaactcggaaagggttctttcaagtatgc
gtgggttcttgacaagctcaaggccgagcgtgagcgtggtatcaccatcgacattgccctctggaagttcga
gactcccaagtactatgtcaccgtcattggtatgtttttggtttcctcattgacatctcgaaaatcattatcctaacg
tgccactctctacagacgctcccggccaccgtgatttcatcaagaacatgatcactggtacctcccaggctga
ctgcgctatcctcattatcgctgccggtactggtgagtcag

Calmodulin (*cal*) sequence(s) in FASTA or text format (Samuels et al. 2006).

taagtctctatatggtcttttgggacgaatctgcaggcggtttgagtgctaatctggtggctacaggacaagga
cggcgatggtacgtagtggctagtgacgcgatactcctctttcccctcctcaccgttgcccctatttgtgcgaat
cattgttggagcacctgaacgtgcgactccgatcgaattttaccgactggcaatttttatgacgaagaaacgga
caaaaaactaacagacgtgatttcgcaggccagattaccaccaaggagctgggcactgtcatgcgctctttg
ggacagaacccctccgagtcagagctgcaggacatgatcaacgaggtcgatgccgacaacaacggatcca
tcgatttccctggtatgtcattaattgaactatttggatggctgccgaaaccgggctaatctagagcggtgaaga
gttccttacc

5.9.9 Trichoderma koningiopis

ITS1 and 2 sequence(s) in FASTA or text format.

tttccgtaggtgaacctgcggagggatcattaccgagtttacaactcccaaacccaatgtgaaccataccaa
actgttgcctcggcggggtcacgcccgggtgcgtcgcagcccggaaccaggcgcccgccggaggg
accaaccaaactctttctgtagtcccctcgcggacgttatttcttacagctctgagcaaaaattcaaaatgaatc
aaaactttcaacaacggatctcttggttctggcatcgatgaagaacgcagcgaaatgcgataagtaatgtga
attgcagaattcagtgaatcatcgaatctttgaacgcacattgcgcccgccagtattctggcgggcatgcctg
tccgagcgtcatttcaaccctcgaacccctccgggggggtcggcgttggggatcgggaaccccctaagacgg
gatcccggccccgaaatacagtggcggtctcgccgcagcctctcctaaacagtgggggcacaactggca
ccgggggggcaatacaaaaacgggggggaaaacgggggaaaccgtggggcaaccaaaagcagagagt
acctagtccc

Translational elongation factor 1-α (*tef1*) sequence(s) in FASTA or text format (Holmes et al. 2004).

atcgagaagttcgagaaggtaagctcatttcactgctttttctaccacgcttggcacaatcgtgtccgacaattc
tgttctcagtcttgtcctgttttcctcgcagcgtcacaccccgcttgacctgtctacccctcctttggcagcaaatt
tttctgctgccttgtttgactatagtggggtaccaatttttttttggcaaccccgctatcgccactgtctctcatccat
cgtcccaacagaatgcactcattcaatcgcatcgtcttttggctcgatttctctatggttcattgtgctgatcatgct
tcaatcaataggaagccgccgaactcggcaagggttccttcaagtatgcgtgggttcttgacaagctcaagg
ccgagcgtgagcgtggtatcaccatcgacattgccctctggaagttcgagactcccaagtactatgtcaccgt
cattggtatgttattcctggcttttgacatgtcgaaatcaccattctaacgtgccactacagacgctcccggtcac
cgtgatttcatcaag

Calmodulin (*cal*) sequence(s) in FASTA or text format (Samuels et al. 2006).

taagtctctagatggtgttttgcgacatatctgcaggcaatatgggtgctaatctggtggctacaggacaagga
cggcgatggtacgtagtggctagtgacgcgatactcctctttcccctcctcaacgttccccttttattttgtgcgaa
ccagtgttggaacacctgaacatgcgacagcgatcgaaattttaccgactggcagttttttcaacgaagaaac
ggacaagaaactaacagatgttgtttcgcaggccagatcaccaccaaggagctgggcactgtcatgcgctct
ttgggacagaaccctccgagtcagagctgcaggacatgatcaacgaggtcgatgccgacaacaacggat
ccatcgatttccctggtatgtcactagctgaaacacttaaactgctaccgaatacgggctaatctagagcggtg
aagagttccttacc

5.9.10 Trichoderma melanomagna

ITS1 and 2 sequence(s) in FASTA or text format.

gcccgccggaggaccaatttacaaactcttttgtatatcccatcgcggattctttacattctgagctttctcggc
gctcctagcgagcgtttcgaaaatgaatcaaaactttcaacaacggatctcttggttctggcatcgatgaagaa
cgcagcgaaatgcgataagtaatgtgaattgcagaattcagtgaatcatcgaatctttgaacgcacattgcgc
ccgccagtattctggcgggcatgcctgtccgagcgtcatttcaaccctcgaacccctccgggggggtcggcg
ttggggatcggcac

Or

tggggcgaccagaagaaggccatgagctcgaccgcaggcgtgtcccaggtgctgaaccgtta-
cacttttgcttcgaccctgtcacatttgcgtcgtaccaacacacccatcggacgagatggtaaactggc-
caagcctcgtcagcttcacaacacgcactggggcttggtctgcccggctgagacgcccgaggga-
caagcttgtggcttggtcaagaacttgtctctgatgtgttatgtcagtgtcggatctccctctgaaccccctgatc-
gagtttatgatcaacagaggcatggaagtcgttgaagaatatgaacctctgcgatatcctcatgccaccaa-
gatctttgtcaacggtgtctgggttggcgttcaccaagatcctaagcacctggtgaaccaagtcttggacac-
gcgtcgcaaatcctatctccagtacgaggtctctctgatcagagacattcgtgatcaggaattcaaaatcttttc-
cgacgcaggtcgtgtcatgcgtcctgtgtttacggtccaacaggaggatgaccccgagacgggcatt-
gagaagggccacttggtcctgacgaaggagctcgtcaacaagttggcaaaggagcag-
gcagagccccccgaagacccgagcatgaaggtcggatgggaagggctcattcgggcgggtgcggtt-
gaatatctcgatgccgaggaagaggagacggccatgatttgcatgacgccggaggatctcgagctctatc-
gtcttcagaaggctggcattgccacggaagaagacatgagcgacgatccgaacaagcgactcaagac-
gaagacgaatcctacgactcacatgtacacgcattgcgagattcacccgagtatgatcttgggcatctgtgc-
tagtatcattcctttc

Translational elongation factor 1-α (*tef1*) sequence(s) in FASTA or text format (Chaverri and Samuels 2003).

agctcatcgtcgccatcaacaagatggacactgccaactgggccgaggctcgttaccaggaaatcatcaag
gagacctccaacttcatcaagaaggtcggcttcaaccccaaggctgttgctttcgtccccatctccggcttcaa
cggtgacaacatgcttgccccctccaccaactgcccctggtacaagggctgggagaaggagaccaaggct
ggcaagtccaccggtaagacccttctcgaggctatcgactccatcgagcctcccaagcgtcccacggacaa
gcctctccgtcttcccctccaggacgtgtacaagattggcggtattggaacagttcccgtcggccgtatcgag
actggtgtcctcaagcccggtatggtcgttacctttgctccctccaacgtcaccactgaagtcaagtccgtcga
gatgcaccacgagcagctcgccgagggccagcccggtgacaacgttggattcaacgtgaagaacgtttcc
gtcaaggaaatccgccgtggtaacgttgccggtgactccaagaacgacccccccaagggcgccgcttctttt
caccgcccaggtcatcgtcatgaaccaccccggccaggtcggtgccggctacgcccccgtcctcgactgc
cacactgcccacattgcctgcaagttcgccgagctccaggagaagattgaccgc

5.9.11 Trichoderma viride

ITS1 and 2 sequence(s) in FASTA or text format.

cggtgggagaccttgggaaggaaccataacgaatttaccacttcccaaccccaagttgacggttaccaaacg
gttccttgggggggccacccccgggttgggtgcaagcccggaacccagccgccgccggaagaaccca
cccaacttctttcggaattcccttggggaagtaatttttttacagctctgagcaaaaattcaaaatgaatcaaaact
ttcaacaacggatctcttggttctggcatcgatgaagaacgcagcgaaatgcgataagtaatgtgaattgcag
aattcagtgaatcatcgaatctttgaacgcacattgcgcccgccagtattctggcgggcatgcctgtccgagc
gtcatttcaaccctcgaacccctccgggggatcggcggtggggatcggggccctcccgcgggcatcggcc
ccgggggcagtcccgaggggacccgggggcatttcaaaagggggggggttcgtgggggaacccgg
gggcaatacaaaaaggggggaatccggagggaacctggggcattcaaaagcgagagtacgtaggcc

Or

aaactgttgcctcggcggggtcacgccccgggtgcgtcgcagccccggaaccaggcgcccgccg-
gagggaccaaccaaactctttctgtagtcccctcgcggacgttatttttacagctctgagcaaaaattcaaaat-
gaatcaaaactttcaacaacggatctcttggttctggcatcgatgaagaacgcagcgaaatgcgataagtaat-
gtgaattgcagaattcagtgaatcatcgaatctttgaacgcacattgcgcccgccagtattctggcgggcat-
gcctgtccgagcgtcatttcaaccctcgaacccctccgggggggtcggcgttggggacttcgggaacccccta-
agacgggatcccggccccgaaatacagtggcggtctcgccgcagcctctcatgcgcagtagtttgca-
caactcgcaccgggagcgcggcgcgtcc

Translational elongation factor 1-α (*tef1*) sequence(s) in FASTA or text format (Holmes et al. 2004).

atcgagaagttcgagaaggtaagctcatttcactgctttttcgctacgcgttcatggcccaatcgaacccgaca
attctgttctcagtcttgtcaacttttccctcgcagcatcacaccccgctttgcctgcctctacccctcattttgcac
agcaaaatttttctggcagtcttgtttggctctgagtggggtgtcaatttttgttggcagcgaccccgccatcgcc
actgtccctcatccatcgtcccaacacattgtgctcattcaatcgcatcgtcttttgcctcaattcctttgtggttca
ttgtgctgatcatgtttcaatcaataggaagccgccgaactcggcaagggttctttcaagtatgcgtgggttctt
gacaagctcaaggccgagcgtgagcgtggtatcaccatcgacattgccctctggaagttcgagactcccag
atactatgtcaccgtcattggtatgtttttggttccctcaatgacatttcgccatcatcattctaacgtgccactctg
cagacgctcccggccaccgtgatttcatcaag

Calmodulin (*cal*) sequence(s) in FASTA or text format (Samuels et al. 2006).

taagtctctagatcgtcttttgcgacaaatctgtaggcgatttgggtgctaatctggtggctacaggacaagga
cggcgatggtacgtagtggctagtgacgcgatactcctctttcccctcctcaccgtccccccctatttgtgcga
accattgctggagcacctgaacctgcgacaccgatcgaaatttcaccgactgggaatttttatggcgaagaaa
cgggcaagaaactaacagacgtgatttcgcaggccagatcaccaccaaggagctgggcactgtcatgcgt
ctttgggacagaacccctccgagtcagagctgcaggacatgatcaacgaggtcgatgccgacaacaacgg
atccatcgatttccctggtatgtctttagctgaaacacatgggcggctgccgaatacgggctaatctagagcg
gtgaagagttccttacc

5.9.12 Trichoderma theobromicola

ITS1 and 2 sequence(s) in FASTA or text format.

atgtgaacgttaccaaactgttgcctcggcggggtcacgccccgggtgcgtcgcagccccggaaccaggc
gcccgccggaggaaccaaccaaactctttctgtagtcccctcgcggacgtatttcttacagctctgagcaaaa
attcaaaatgaatcaaaactttcaacaacggatctcttggttctggcatcgatgaagaacgcagcgaaatgcg
ataagtaatgtgaattgcagaattcagtgaatcatcgaatctttgaacgcacattgcgcccgccagtattctggc
gggcatgcctgtccgagcgtcatttcaaccctcgaaccctccgggggatcggcgttggggatcgggaccc
ctcacacgggtgccggccccgaaatacagtggcggtctcgccgcagcctctcctgcgcagtagtttgcaca
actcgcaccgggagcgcggcgcgtccacgtccgtaaaacacccaacttt

Translational elongation factor 1-α (*tef1*) sequence(s) in FASTA or text format (Samuels et al. 2006).

taagctcactttcactgctttttcccacgcatttttggcacaattgtgtccgacaattctgtttatcagtcttgtcaat
cttttctctctcccagcatctcaccccgctttgcccttctacccctcctttggcacagcaaaaaattttctggctgc
cttgtttggcttttagtggggtgtaatttttttttggcagcaaccccgctatcgccactgtccctcatccactgccc
aacagattttacttgctcagtcgtatcgtctgttctcaaatttctttcggttcattgtgctgatcatgcttcaatcaat
aggaagccgccgaactcggcaagggttccttcaagtatgcgtgggttcttgacaagctcaaggccgagcgt
gagcgtggtatcaccatcgacattgccctctggaagttcgagactcccaagtactatgtcaccgtcattggtag
gttttcagtcctcgtgctacttgacatgtcaaaatcatcaatctaacatgttgctctacagacgctcccggtcacc
gtgatttcatcaa

Calmodulin (*cal*) sequence(s) in FASTA or text format (Samuels et al. 2006).

taagtctctagatactcttttgcgataattcttcaagcgatttggggtactaatatggtggctacaggacaaggac
ggcgatggtacgtagtggctagtgacgcgatactcctctttcccctcctcaccgtccccctattttgtgcgagct
attaccagagcgcctgaac ctgcgataccgatcgaaatttccccggctggcaatttttatgacg
aatacaaggacaagaaactaacagacttgacttcgcaggccagatcaccaccaaggagctgggcactgtca
tgcgctctttgggacagaacccctccgagtcagagctgcaggacatgatcaacgaggttgatgccgacaac
aatggatccatcgatttccctggtatgtcaatagcagaaacacatggacggctgccgaatacgggctaatcta
gagcggtgaagagttccttacc

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