Ajit Varma · Arun Kumar Sharma *Editors*

Modern Tools and Techniques to Understand Microbes

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Foreword

Microbiology is one of the leading branches of modern biology and is interdisciplinary in scope as it interfaces with various basic and applied disciplines of science, including but not limited to biology, chemistry, physics, agriculture, medicine, engineering, and environmental/atmospheric sciences. Consequently, the study of microbiology has been divided into several subspecialties such as agricultural microbiology, medical microbiology (bacteriology, virology, mycology, and parasitology), food/dairy microbiology, soil microbiology, environmental microbiology, microbial ecology, and microbial biotechnology, among others. The commonality among these subspecialties is the study of microorganisms, including prokaryotes (bacteria and viruses), eukaryotes (yeasts, fungi, algae, and protozoa), and archaea, which essentially requires the use of overlapping basic tools and techniques.

Microorganisms interact with both living beings (plants, animals, and host microbes) and non-living matter (natural and engineered materials and environments including soil, water, and air) in useful or harmful associations. Consequently, microorganisms are studied for understanding their role in causing infections/diseases in humans, animals, and plants as well as for mediating various industrial/biotechnological and natural processes important for sustaining and improving life on this planet. Except for an estimated 1 % of microbes which cause disease, microorganisms contribute positively to improve various facets of human life. Notably, microbes have been harnessed for development of new products including medicines (such as antibiotics, probiotics, antioxidants, and herbal products), agro-fertilizers, agro-pesticides, biocides and biocontrol agents, biosurfactants, bioplastics, specialty chemicals, and fermentation-based products of brewery and dairy/food industries. In the modern-day economic scenario, understanding and manipulation of new genes and proteins dominate the intended role of microorganisms; however, the cellular environment must be optimized for efficiency, practicability, and production costs in the creation of new products. In other contexts, addressing of pressing topics in modern microbiology, such as the escalating concerns on antimicrobial resistance and the need for characterization of microbial communities and microbiomes, requires the understanding of OMICs

approaches (genomics/metagenomics, proteomics, and metabolomics) for microorganisms, their hosts, and host–microbe interactions.

Historically, human survival and sustainability have depended on the unavoidable natural resource for mankind, the plant kingdom. Beneficial interactions between the plants and microbes have been known to play a crucial role in the establishment of this resource in a given ecosystem. However, there is incomplete understanding of the microbial types critical in such symbioses, although those developing preferentially or exclusively in roots, like rhizospheric and rhizoplane microbes, and on leaves, such as phyllospheric and phylloplane microbes, must play a key role.

For the aforementioned reasons, it has become of crucial importance to assemble the ever-improving and newly emerging methods of modern microbiology in a comprehensive book (such as this compilation) relevant for immediate use in the laboratory as well as for pursuing the practical applications of microbiology. The covered topics essentially include cutting-edge techniques of molecular biology, immunology, microbiology, and structural biology as applied to modern microbiology; the complexity of the systems involved ranges from individual molecules to prokaryotic organisms to eukaryotic organisms, with a focus on bacteria, fungi, and related plant host aspects. Furthermore, this book has been authored by experts from different subspecialties of modern microbiology to facilitate the extremely difficult task of compiling the state-of-the-art methods covering diversified topics of interest to modern microbiologists. The 27 chapters are indeed outstanding contributions on important topics in the field. The comprehensiveness should make the volume equally valuable to students, teachers, and enthusiastic researchers entering or pursuing the field of microbiology, as a "go-to" reference book as well as a resource to access expert laboratories and scientists in various specializations. Considering the state-of-the-art nature of the contents, this volume is expected to be immensely useful for a long time to come.

Overall, this book is an excellent compendium on modern tools and techniques in the most usable form on the frontline topics in microbiology. I wish to extend my congratulations and best compliments to the editors of the book for their outstanding efforts in obtaining valuable contributions from a team of global experts. The book is a timely and cutting-edge compilation on modern aspects of a subject (microbiology) which has wide-ranging major implications across the globe including, but not limited to, the importance for food security worldwide as well as for ameliorating the socioeconomic condition of communities affected by climate change at the grass roots of human society.

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Preface

It is our consensus that many new methods and approaches are now available to gain access to the microorganisms residing in an ecosystem that allow better assessments of microbial diversity. Species diversity consists of species richness, the total number of species present, species evenness, and the distribution of species. Methods to measure microbial diversity in any ecosystem can be categorized into morphological/biochemical (phonetic) and molecular (genetic) based techniques. Some of the molecular techniques provide characteristics that are useful to discriminate among communities without providing any information about the types composing the community, e.g., DNA hybridization, percent G+C, DNA reassociation, restriction fragment length polymorphism (RFLP), and community-level physiological profiles (CLPP). A variety of DNA fingerprinting techniques by employing polymerase chain reaction (PCR) can be used to rapidly differentiate closely related environmental stain, viz., arbitrarily primed (AP-PCR), repetitive (REP-PCR), denaturing/temperature-gradient gel electrophoresis (DGGE/TGGE), terminal-restriction length polymorphism (T-RFLP), amplified ribosomal DNA-restriction analysis (ARDRA), etc. Researchers argued that measures of diversity are pointless because the dynamic nature of the microbial world meant that communities did not have a characteristic diversity but changed as the environment changed.

The relationship between structure and function in a community can only be understood, predicted, and engineered through an understanding of the source of diversity from which the community is drawn. Based on polyphasic approaches, prokaryotic taxonomy is generally taken as a synonym of systematics or biosystematics and is traditionally divided into three parts: (i) *Classification*, i.e., the orderly arrangement of organisms into taxonomic groups on the basis of similarity; (ii) *Nomenclature*, i.e., the labeling of the units defined in (i), and (iii) *Identification* of unknown organisms, i.e., the process of determining whether an organism belongs to one of the units defined in (i) and labeled in (ii).

Species diversity consists of species richness, the total number of species present, species evenness, and the distribution of species. Methods to measure

microbial diversity can be categorized into two approaches: biochemical-based techniques and molecular-based techniques. Typically, diversity studies include the relative diversities of communities across a gradient of stress, disturbance, or other biotic or abiotic differences. It is difficult with current techniques to study diversity since we do not know what is present and we have no way of determining the accuracy of our extraction or detection methods. An attempt has therefore been made to reduce the information gathered by diversity studies into discrete, numerical measurements such as diversity indices.

Traditionally, diversity was assessed using selective plating and direct viable counts. These methods are fast and inexpensive and can provide information on the active, heterotrophic component of the population. Limitations include the difficulty in dislodging bacteria or spores from soil particles or biofilms, growth medium selections, growth conditions (temperature, pH, light), inability to culture a large number of bacterial and fungal species with current techniques, and the potential for colony–colony inhibition or of colony spread. In addition, plate growth favors those microorganisms with fast growth rates and those fungi that produce large number of spores. All of these limitations can influence the apparent diversity of the microbial community.

A biochemical method that does not rely on culturing of microorganisms is fatty acid methyl ester (FAME) analysis. This method provides information on the microbial community composition based on grouping of fatty acids. It has been used to study microbial community composition and population changes due to chemical contaminants and agricultural practices. PCR targeting the 16S rDNA has been used extensively to study prokaryote diversity and allows identification of prokaryotes as well as the prediction of phylogenetic relationships. These are two similar methods for studying microbial diversity. These techniques were originally developed to detect point mutations in DNA sequences. DGGE can separate DNA with one bp difference, whereas TGGE uses the same principle as DGGE except that the gradient is temperature rather than chemical denaturant (formamide and urea). DGGE/TGGE has been used to assess the diversity of bacteria and fungi in the rhizosphere, caused by changes of nutrient addition and addition of anthropogenic chemicals. While the rRNA genes have been the main target of microbial diversity studies using DGGE, some researchers have targeted catabolic genes, such as methane monooxygenase for DGGE analysis. Besides above approaches, there are some other approaches which can be used for the identification of microbial diversity. Bacterial phylogenetic classification is based on sequence analysis of the SSU 16S rRNA molecule or its genes. Over 20,000 SSU RNA gene sequences have now been deposited in specialist r-RNA databases such as the rRNA Database Project (RDP).

It is important to study microbial diversity not only for basic scientific research but also to understand the link between diversity and community structure and function. Although methods to study diversity (numerical, taxonomic, structural) are improving for both bacteria and fungi, there is still not a clear association between diversity and function. Even if an organism is functionally redundant in one function, chances are it is not redundant in all functions and will have different

susceptibilities and tolerances to abiotic and biotic stresses. It is generally thought that a diverse population of organisms will be more resilient to stress and more capable of adapting with environmental changes. Methods to understand the link between structural diversity and functioning of below- and aboveground ecosystem need to be developed so that the question of how diversity influences function can be addressed.

This volume has been designed to enumerate microbial community in an ecosystem by using phenetic and genotypic approaches. The volume comprises 26 chapters dealing with the most recent and modern tool, techniques, and protocol. It was prepared in such a manner that any novice can follow with ease and conduct the experiment for conclusive results. We are very grateful to contributors for their great efforts in the compilation of such informative book that will be useful to researchers and further dissemination of knowledge. Their patience and support is highly appreciative. The volume editors are thankful to Dr. Jutta Lindenborn and Dr. Hanna Hensler-Fritton.

Ajit Varma
Arun Kumar Sharma

Contents

Chapter 1
Introduction to Modern Tools and Techniques to Understand Microbes

Pankaj Goyal, Diksha Bhola, and Ajit Varma

Abstract Microorganisms are omnipresent in the nature and having both positive and negative effects on various aspects of human life and society. To study their impact on environment and other associated fields, it is crucial to be able to cultivate the microbes under laboratory conditions and to detect and identify them quickly and accurately. However, difficulties in cultivating most of the microbes by standard traditional methods such as plate count, filteration, etc., limit our ability to study these tiny organisms. To enhance the microbial studies effectively particularly for microbes which are viable but non-culturable (VBNC), several modern approaches such as molecular methods are proving to be appreciated. The most appreciable modern molecular methods which are used to detect and identify organisms include rRNA or rDNA fingerprinting, probing, and sequencing.

1.1 Introduction

Microorganisms are integral part of human life and various aspects of daily needs. Although these organisms are very tiny in size, however, their impact on society is very large, and they play very crucial role in both positive and negative effects. Although these tiny organisms are ubiquitous in nature, major sources are water, soil, and air from where they spread almost everywhere. Our little knowledge to study microbes in natural environments does not fulfill the requirement, because of the fact that studying microbial diversity in nature is not an easy assignment. Traditionally used culturing techniques do not give rise to much information as required in the current scenario of the microbial world. Microbes exist in very high numbers in natural environment; however, several thousands of microbial species have not yet been characterized so far because of lack of proper and modernized tools and techniques to understand these microbes.

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The conventional methods for detecting and identifying bacteria from different types of samples are based on culturing, enumeration, and isolation of presumptive colonies for further identification analysis. Microscopy and culture techniques have been used widely today; however, they provide a limited view of the microbial world. Many microbes look similar under a microscope, and many will not grow outside of their natural environments. Due to the limited availability of modernized tools, only less than 1 % of all microbial species have been identified so far. This raised a million dollar question regarding these viable but non-culturable microbes (VBNC), i.e., How many are there? Who are they? And what are they doing? Thus, early microbiologists were only able to study a small number of microbes—those that grew in the laboratory (Kell et al. 1998). Microbes usually adapt to almost all the different environments that exist on the earth from normal to extreme environment like polar or desert region as well. And they are able to perform various functions including decomposing the chemical components made by other living organisms. Here comes the fact to address some important conclusions of studying bacteria in their natural environment and further to know their functions as well as the qualitative variations in different types of commodities. In order to understand the exact microbial phenomenon, there is a need to answer these questions and to pursue further studies on basic knowledge regarding the community structure. Modern tools and techniques including molecular analysis are helping scientists to answer some of these questions, and of course, these will be beneficial for the society in order to reveal novel biochemicals useful to humans as medicines, biofuels, and many more.

1.2 Motives for Studying Microbial Diversity

Different hypothesis suggests that culturable bacteria represent only a minor fraction of the total bacterial population present; however, a large amount of genetic information is yet to be discovered because of the VBNC nature of microbes present in natural environment (Giovannoni et al. 1990). It is also important to continue the research both on the culturable and the VBNC microbes by utilizing modern tools and techniques so that their hidden properties could be revealed. It will also give a glimpse about the extant and extinct microbes because of the fact that there is no consensus on how many species exist in the world, the potential usefulness of most of them, or the rate at which they are disappearing or emerging (Fakruddin and Mannan 2013).

Studying microbial diversity is also essential because of the following facts:

- To increase the knowledge of the diversity of genetic resources
- To understand the distribution of organisms
- To obtain the knowledge of the functional role of microbial diversity
- To understand the regulation of biodiversity
- To understand the magnitudes of biodiversity

1.2.1 Factors Affecting Microbial Diversity (Zhao et al. 2012)

Majorly two groups:

1.2.1.1 Abiotic Factors

Abiotic factors include both physical and chemical factors such as water availability, salinity, oxic/anoxic conditions, temperature, pH, pressure, chemical pollution, heavy metals, pesticides, antibiotics, etc.

1.2.1.2 Biotic Factors

Biotic factors include plasmids, phages, transposons, etc., that influence genetic properties and phenotypes of their host.

1.3 Tools and Techniques to Understand Microbes

Prokaryotic organisms are classified on the basis of various characteristics such as morphological characteristics, viz., cell shape, cell wall, movement, flagella, Gram staining, etc.; however, these systems may not be adequate for establishing a detailed classification of microbes. Recent advances in molecular biology have provided a promising alternative in estimating microbial diversity without having to isolate the organisms (Giovannoni et al. 1990).

1.4 Traditional Methods

Traditional methods including biochemical analysis and multivariate data analyses are having high significance to study the microbes along with their physiological diversity. Traditional culture methods are time-consuming and laborious; however, the isolation and purification of microorganisms allow for further subtyping analysis and for storage in culture collections. The more conventional methods for further subtyping of bacteria include the study of the phenotypic characteristics of the microorganisms. In order to distinguish between different types of microbes, early microbiologists studied metabolic properties such as utilization of different carbon, nitrogen, and energy sources in addition to their requirements for growth factors. These phenotypic methods include biotyping, serotyping, and phage typing (Arbeit 1995). In biotyping, the biochemical growth requirements, environmental

conditions (pH, temperature, antibiotic resistance, bacteriocins susceptibility), and physiological (colony and cell morphology, cell wall composition by microscopy, and membrane composition such as by fatty acid analysis) aspects of bacteria (Vanda me et al. 1996) are investigated, while serological and phage typing (Towner and Cockayne 1993) concentrate more on the surface structure differences of bacteria. Phages are also very useful in subtyping of bacterial species from different types of samples (Hagens and Loessner 2007; Kretzer et al. 2007). The following methods are prominently used to study microbes under in vitro conditions.

1.4.1 Total Microbial Plate Counts

This is the most traditional method for assessment of microbial diversity by both selective and differential plating and subsequent viable counting. Being fast and inexpensive, these methods provide information about active and culturable heterotrophic segment of the microbial population. These methods select microorganisms with faster growth rate and fungi producing large number of spores. There are several factors that limit the use of these methods such as difficulties in dislodging bacteria or spores from soil particles or biofilms, selecting suitable growth media, provision of specific growth conditions (temperature, pH, light), inability to culture a large number of bacterial and fungal species using techniques available at present, and the potential for inhibition or spreading of colonies other than that of interest. Further, these methods cannot reflect the total diversity of microbial community (Tabacchioni et al. 2000).

1.4.2 Sole-Carbon-Source Utilization

Sole-carbon-source utilization (SCSU) is also known as community-level physiological profiling (CLPP) system (Garland and Mills 1991) and was initially developed to identify pure cultures of bacteria to the species level, based upon their metabolic characteristics. This method gives a significant approach to study both functional and taxonomic perspectives for future research on microbial communities from contaminated sites, plant rhizospheres, arctic soils, soil treated with herbicides, etc., SCSU has an advantage in its ability to differentiate between microbial communities, relative ease of use, reproducibility, and production of large amount of data describing metabolic characteristics of the communities. However, this method can detect only culturable and slow-growing microbes.

1.4.3 Phospholipid Fatty Acid Analysis

This method is also known as the fatty acid methyl ester (FAME) analysis. Fatty acid composition (mainly C2–C24) has been used significantly to characterize microorganism(s) (Banowetz et al. 2006). The fatty acid composition is comparatively stable and is independent of plasmids, mutations, or any sort of damaged cells. The method is inexpensive, quantitative, and robust and has high reproducibility. However, bacterial growth conditions are sometimes reflected in the fatty acid pattern. It is also not possible to detect individual strains or species of microorganisms, but changes in the overall compositions of the community can be detected. Furthermore, cellular fatty acid composition can be influenced by temperature and nutrition, and individual fatty acids cannot be used to represent specific species because individuals can have numerous fatty acids, and the same fatty acids can occur in more than one species.

1.4.4 Light Microscopy

A variety of light microscopes are employed by biologists to analyze organisms throughout. Depending upon their application, different types of light microscopy are used to study samples. The most commonly used light microscopes are the bright field, dark field, fluorescent, and confocal microscopes.

1.4.4.1 Bright Field Microscope

A bright field microscope is also called as a compound microscope and consists of a supporting system which includes a base, body, and stage, illuminating system of a concave mirror along with a condenser and a magnifying system having optical lenses. The specimen to be examined appears dark in contrast to a brightly illuminated microscopic field. Thus, it is called the bright field microscope (Fig. 1.2a). The magnification power of these microscope ranges between 1000× and 2000× (Pelczar et al. 1998). However, resolving power is the basis limitation of a bright field microscope.

1.4.4.2 Dark Field Microscope

In contrast to a light field microscope, the dark field microscope provides an image of a brightly illuminated sample against a dark background (Pelczar et al. 1998). Dark field microscope is used under circumstances where an unstained section or a living sample is to be examined. This type of microscope has a special condenser that only allows the transmission of a hollow cone of light (Fig. 1.1).The light

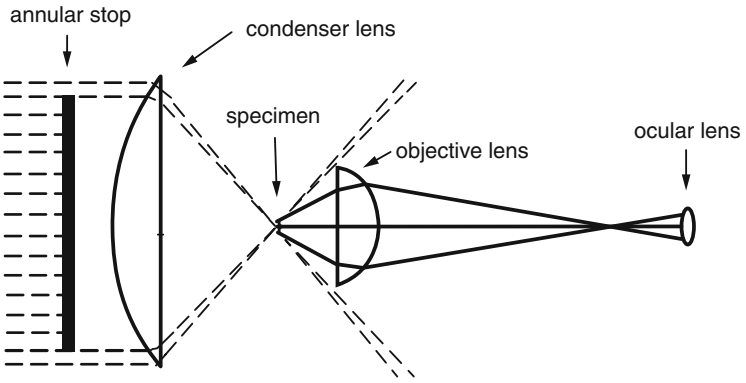

Fig. 1.1 Ray diagram for dark field microscope (source: www.gonda.uda.edu)

transmitted does not fall on the objective piece, thus giving a dark background. However, the living samples diffract some of the rays of light which leads to these samples appearing bright against a dark background.

1.4.4.3 Fluorescence Microscope

The ability of certain substances to emit light of higher wavelength when exposed to ultraviolet or blue–violet light is called as fluorescence. Further, these substances are known as fluorochromes (Prescott et al. 2005). In fluorescence microscopy, the samples to be studied are stained with fluorochromes and illuminated with violet and ultraviolet light. The fluorescent light emitted by the sample is perceived by the eye and the sample appears bright green against a dark background. A specialized filter is present after the objective lens; it eradicates any residual UV light present which might be harmful to the eyes of the viewer. It also eliminates blue or violet light which may reduce the contrast of the image (Fig. 1.2c).

The most commonly used dyes for staining in fluorescent microscopes are *auramine*, *acridine orange*, *berberine sulfate*, *primuline*, *thioflavines*, *trypaflavine*, *morin*, *rhodamine*, etc. Fluorescent microscope is an indispensable instrument for study in the field of medical microbiology as well as microbial ecology. With the help of fluorescent microscope, microorganisms can be easily viewed and counted directly while keeping the ecological niche relatively undisturbed.

1.4.4.4 Phase Contrast Microscope

This kind of microscopy was first developed in 1953 by Frederick Zernike, thus called as Zernike microscope. These microscopes are extensively used in applied and theoretical biology studies such as studying unstained living cells and their internal structures, determining microbial motility, and detecting endospores,

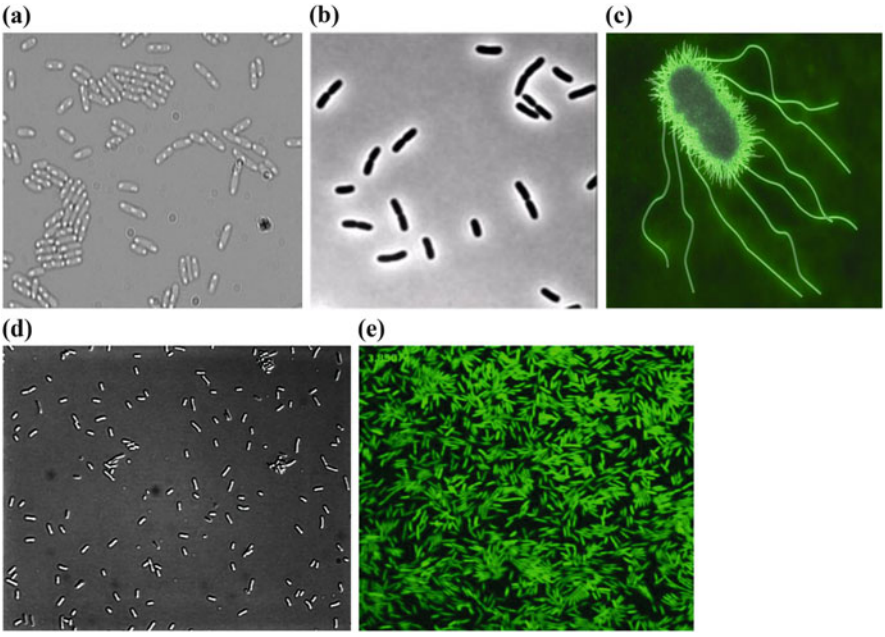

Fig. 1.2 Microscopic view of *E. coli* (**a**) bright field microscope (source: www.rpgroup.caltech.edu), (**b**) phase contrast microscope (source: physweb.bgu.ac.il), (**c**) fluorescent microscope (source: www.nrc-cnrc.gc.ca), (**d**) differential interference contrast microscope (source: www.lookfordiagnosis.com), (**e**) confocal microscope (source: commons.wikimedia.org)

inclusion bodies, etc.; this microscope is based on the principle that even a slight change in refractive index of the material causes the light passing through it to undergo a phase change (Zernike 1942). These different phases of the light passed from a contrasting image and thus help in studying the internal structure of an unstained sample (Fig. 1.2b). The phase contrast microscope operates on a special system consisting of phase condenser and objective. This system enables the study of an unstained living structure based only on their thickness or change in refractive index. The ability to study differences in the internal structure of a cell is the advantage phase contrast microscope has over other microscopic methods.

1.4.4.5 Differential Interference Contrast Microscope

DIC or the stereo microscope produces an image analyzing the differences in thickness and refractive indices of the sample as in case of phase contrast microscope (Prescott et al. 2005). However, in this microscope, two beams of polarized light which are at right angles to each other are used. The two beams interfere and combine to give a three-dimensional image of the sample (Fig. 1.2d). Using differential interference contrast microscope, unstained living specimens such as

nuclei, granules, cell walls, vacuoles, and endospore can be clearly viewed in brightly colored three-dimensional images.

1.4.4.6 Confocal Microscope

As the interest in studying processes occurring at cell level increased, there also increased the need for a sophisticated technology that would facilitate in vivo studies. Thus, in 1957, Marvin Minsky produced a confocal microscope. All the modern microscopes are based on the principle implied by him (Minsky 1988). All the biological samples are three-dimensional, and confocal microscopes effectively study these samples as compared to the traditional microscopes which form hazy images of these samples. Laser beam is used by the confocal microscope to illuminate a fluorescently stained sample. In confocal microscope, an aperture above the objective lens eliminates stray light from the parts of the specimen which are not in the plane of focus thus creating a sharper image. The information produced from each plane of the specimen is perceived by the computer attached to the microscope. This information is used by the computer to form a detailed three-dimensional image of the specimen (Fig. 1.2e). Thus, this highly sophisticated and modern tool has varied applications which include morphological studies. The wide range of applications include morphological studies of varied cells and tissues, neuroanatomy and neurophysiology studies, analysis of fluorescent proteins, photo-bleaching studies, research in stem cell, DNA hybridization analysis, multiphoton microscopy, and many more. This microscope is also highly competent in the study of biofilms.

1.4.5 Electron Microscopy

Light microscopes have been used for decades to study microorganisms. However, even the most sophisticated light microscopes have about 0.2 μm resolution limit. This greatly reduces their effectiveness in studying many microorganisms. These limitations lead to the development of electron microscope. Electron microscopes have much higher resolution attributing to use of a beam of electrons rather than a beam of light.

1.4.5.1 Transmission Electron Microscope

In TEM, a beam of electron interacts and passes through a specimen. Electrons which have much shorter wavelength in comparison to ordinary light provide thousand times greater magnification than light microscope (Voutou and Stefanaki 2008). A tungsten filament acts as electron source. The electrons emitted by it are focused and magnified via a system of magnetic lenses. This electron beam passes

through the condenser aperture and falls on the sample. Some of the electrons are scattered by the sample; others that pass through it are used to form an image on a fluorescent screen. Denser region of the sample scatters more electrons and appears dark in comparison to lighter region. The image produced can be seizure digitally or using photographic sheet (Fig. 1.3b). In TEM the sample must be analyzed under vacuum conditions as the electrons get deflated by air molecules. This feature restricts TEM and only extremely thin sample can be viewed. Further, the specimens are prepared for analysis using specialized techniques such as mechanical thinning, ion thinning, negative staining (Woeste and Demchick 1991), and shadowing. Freeze-etching is another technique used in TEM to study three-dimensional intracellular structure of a sample. It is quintessential for various studies in the field of life science, forensic analysis, nanotechnology, gemology, and metallurgy.

1.4.5.2 Scanning Electron Microscope

SEM functions in a manner which is different from the TEM. In scanning electron microscope, the image is produced when atoms on the surface of the specimen release electrons after receiving a beam of electrons (Voutou and Stefanaki 2008). This narrow beam of electrons is passed throughout the specimen. The secondary electrons released by atoms on the surface are perceived by a detector. In the detector, electrons strike a scintillator producing an electric signal. This is further transferred to a cathode ray tube where an image similar to a television picture is produced. The scanning electron microscope generates a three-dimensional image of the surface of a microorganism (Fig. 1.3a). When the beam of electron falls on the raised area of the specimen, secondary electrons in large number are emitted. However, less number of electrons are emitted when electron beam falls on a depression in the surface. Thus, the raised areas appear to be light and depression appears to be darker on the screen. It is useful in the surface studies of various

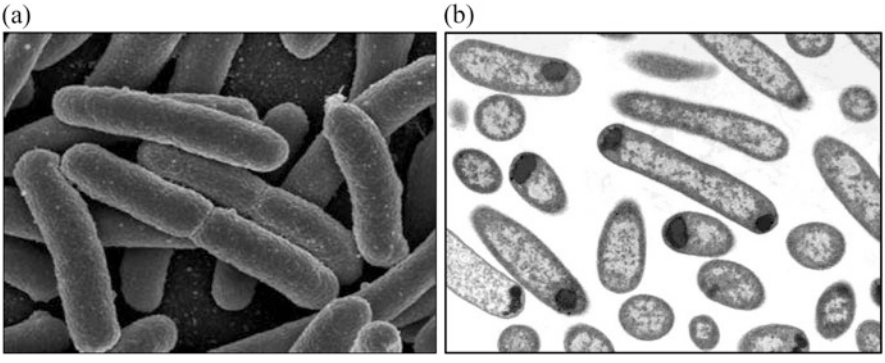

Fig. 1.3 Microscopic view of *E.coli* (**a**) scanning electron microscope (source: schaechter.asmblog.org), (**b**) transmission electron microscope (source: www.oulu.fi)

microbes. It can be effectively used to study location of microorganisms in their various ecological niches. However, SEM provides limited information about crystallography and resolution.

1.4.5.3 Scanning Probe Microscopy

This microscope was developed by Gerd Binnig and Heinrich Rohrer, who shared the Noble Prize for their work in 1986. This microscope is one of the most powerful modern microscopes developed in recent times. This microscope analyzes the surface of the specimen by moving a needle thin probe over the surface. By this, it achieves a maximum magnification of hundred million. When a minute voltage is applied between the tip of the probe and the specimen, electrons move along a narrow channel resulting in a tunneling current. The movement of the probe on the surface of the specimen is detected and analyzed digitally to produce a precise three-dimensional image of the specimen. Since the microscope can analyze specimens immersed in water, scanning probe microscope is extensively used in studying biological molecules (Prescott et al. 2005).

1.5 Identification of Microbes

1.5.1 Simple Staining

Microorganisms can be readily stained using single dyes. This simple and easy-to-perform procedure is known as simple staining. The specimens are stained for a prefixed time using basic stains such as methylene blue and crystal violet. The excess stain is washed-off and the specimen is studied under the microscope. Simple staining gives us an idea about the arrangement pattern, shape, and size of the bacteria.

1.5.2 Differential Staining

Differential staining is the staining technique where microorganisms are demarcated on the basis of their staining properties (Prescott et al. 2005).

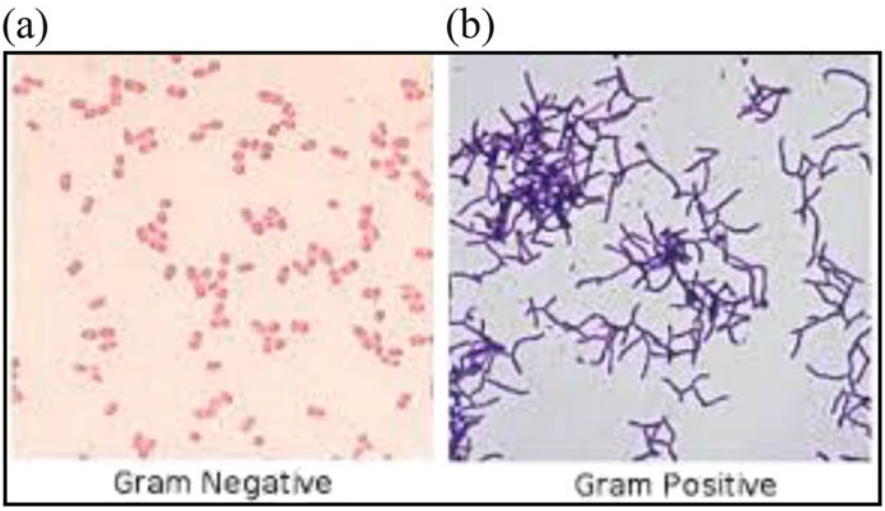

Fig. 1.4 Gram staining (source: water.me.vccs.edu) (**a**) Gram-negative bacteria (**b**) Gram-positive bacteria

1.5.3 Gram Staining

Gram stain was developed by Christian Gram in 1884. It is the most extensively used method of staining in bacteriology. The procedure of Gram staining starts with staining the bacterial smear with crystal violet. Further, iodine solution is added which enhances the retention of dye by the cells. After this, ethanol is added to the smear for decolorization. This is the differentiating step of Gram staining. Finally, the smear is counterstained with safranin. The Gram-negative bacteria appear (Fig. 1.4a) pink to red, whereas the Gram-positive bacteria appear purple (Fig. 1.4b).

1.5.4 Acid-Fast Staining

This is another type of differential stain that is used for the identification of acid-fast organisms such as *Mycobacteria*. Certain organisms like *Mycobacteria* are not easily stained due to high content of lipid particularly mycolic acid in the cell wall. The length of carbon chain present in the mycolic acid of any particular species determines the acid fastness property of that species (Lyon 1991). Thus, harsh treatments, i.e., a mixture of fuchsin and phenol along with heating, are employed. The cells are primarily stained by fuchsin and phenol along with heating. These cells are then decolorized by acid–alcohol and counterstained using methylene blue. The acid-fast cells remain red as they do not decolorize, whereas the nonacid-fast cells decolorize, take up methylene blue, and appear blue. This method

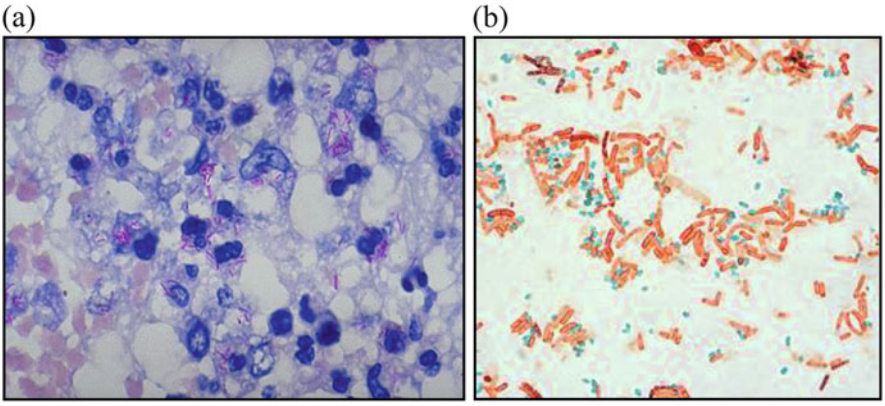

Fig. 1.5 Staining techniques (**a**) acid-fast stain of *Mycobacterium tuberculosis* (source: library.med.utah.edu), (**b**) endospore staining, *Bacilli* endospores *stained green*, and vegetative cells *stained red* (source: www.studyblue.com)

is specifically applied to identify *Mycobacterium tuberculosis* (Fig. 1.5a) as well as *Mycobacterium leprae*.

1.5.5 Endospore Staining

Endospores are the resting structures characteristic of some bacterial genera. These are produced under unfavorable conditions and have high resistance against damage. Thus, it is difficult to stain them, but once stained they do not decolorize easily. Due to the tough nature of endospore, heat is used in facilitating staining of endospores by dyes. One of the examples is Schaeffer–Fulton method of endospore staining. In this procedure, endospores are first stained using malachite green along with heating. After this, the cells are rinsed with water and counterstained using safranin. This procedure gives red to pink cells containing green colored endospores (Fig. 1.5b).

1.5.6 Negative Staining

Negative staining is a technique where the background is stained whereas the specimen does not take up stain. The bacteria are stained with nigrosin dye or India ink and air-dried (Prescott et al. 2005). The bacteria appear light against darkly stained background (Fig. 1.6b). This technique is readily used to study the presence of capsules which surround the bacteria. Also negative staining can be used to study aqueous lipids such as lamellar liposomes (YashRoy 1990).

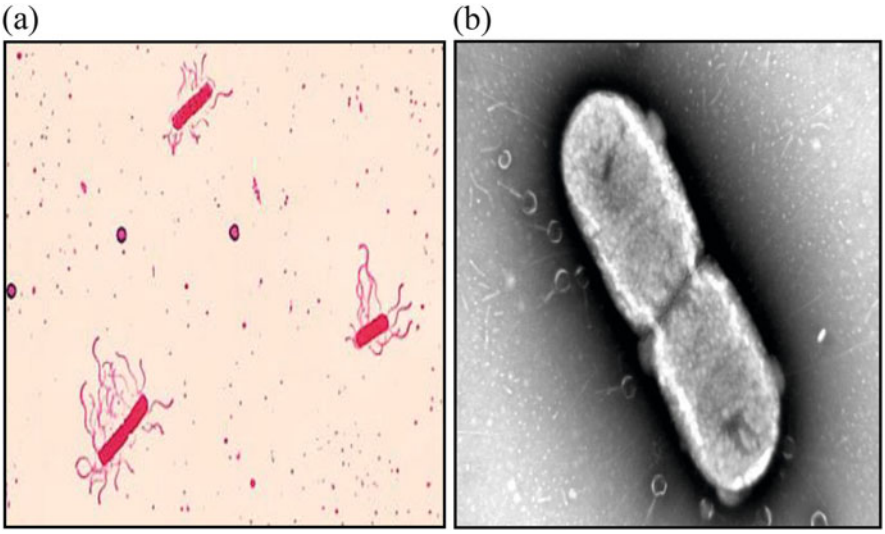

Fig. 1.6 Staining techniques (**a**) flagella staining, *Bacillus cereus* stained with Leifson stain (source: clinical-laboratory.blogspot.com) (**b**) negative staining, intestinal bacterium, and bacteriophages of a bush dog with enteritis (source: www.izw-berlin.de)

1.5.7 Flagella Staining

Flagella are locomotory structures present in organisms and can only be viewed by electron microscope (Prescott et al. 2005). In order to observe them under light microscope, the flagella are coated with mordants such as tannic acid and potassium alum and further stained. The flagella can be stained by gray method involving basic fuchsin (Gray 1926) or by Leifson method which uses pararosaniline (Fig. 1.6a) (Leifson 1930). Since flagella are characteristic feature of the organism, staining methods produce valuable taxonomic information.

However, these phenotypic typing methods are limited since microorganisms are capable of suddenly altering their phenotypic characteristics due to environmental changes or genetic mutations. Therefore, identification by genotypic characteristics has been developed to avoid these problems that can occur with phenotypic methods.

1.6 Modern Molecular Methods

Conventional methods for microbial characterization as described earlier are mainly based on analysis of the culturable microorganisms. However, due to the presence of viable but non-culturable (VBNC) nature of major portion of bacteria from natural communities, the interpretation of overall structure of the community

is very problematic (Dokic et al. 2010). Modern methods including molecular and phylogenetic aspects to characterize microbial diversity are based on genetic diversity and do not require cultivation. Molecular methods used to describe microbes include DNA–DNA and RNA–DNA hybridization, DNA cloning and sequencing, and other PCR-based methods such as denaturing gradient gel electrophoresis (DGGE), temperature gradient gel electrophoresis (TGGE), ribosomal intergenic spacer analysis (RISA), and automated ribosomal intergenic spacer analysis (ARISA).

1.6.1 Polymerase Chain Reaction

Polymerase chain reaction (PCR) (Saiki et al. 1985) is the amplification of a nucleic acid target sequence. The targeted sequences can be a specific gene, repetitive areas in the sequence, or arbitrary sequences. 16S rRNA gene is a favorable PCR amplification target by universal or species–strain-specific primers for identification and phylogenic purposes since it is universally distributed among bacteria, and it contains enough variations among strains and species within the DNA sequence. SYBER Green I and SYBER Gold are nonspecific dyes that bind to the double-stranded DNA of the PCR product (Glynn et al. 2006). Only fluorescent probes labeled with different reporter dyes can be used to detect multiple amplicons within the same reaction mixture, while double-stranded DNA dyes are limited to a single product per reaction. PCR-based techniques have also been developed for screening of genetically modified organisms and their derived materials (Holst-Jensen et al. 2003). Post-PCR detection methods vary from gel electrophoresis, hybridization analysis, and usage of specific nucleic acid probes.

Depending upon the type of samples, different variations of PCR have been developed in order to fulfill simultaneous detection of multiple bacteria, quantification, and differentiation of viable bacterial cells. Multiplex PCR systems have been developed for differentiation of multiple species belonging to single genera and for differentiation of mixed bacterial pathogens (Touron et al. 2005). Conventional PCR is not able to indicate if the bacterial cells are viable or dead, and therefore reverse transcriptase PCR was developed for the specifically detecting viable cells. This method is based on the reverse transcriptase enzyme that is able to use messenger RNA as a template for synthesizing single-stranded DNA in the 5′ to 3′ direction (Rodríguez-Lázaro et al. 2007). The technique is sensitive and do not require pre-enrichment steps, which decreases the time of analysis. Another advantage of the reverse transcriptase PCR is the detection of VNC cells that are not detected with culturing. Quantitative PCR (Monis and Giglio 2006) is based on the monitoring of formation of PCR product simultaneously as the reaction occurs by using fluorescent probes or dyes that are sequence specific or nonspecific. PCR protocols need to be synchronized between laboratories, so that the PCR results are reliable and reproducible when performed in different locations or times. However, this is significant by several aspects including quality of the DNA template,

humidity, chemical and microbiological cleanliness, temperature, equipment, personal practice, and the reaction conditions and the reaction materials (Malorny et al. 2003). Some samples may contain substances that can degrade the target nucleic acid sequence or inhibit the enzyme activity in the PCR, which can give false-negative results (Glynn et al. 2006).

1.6.2 Mole% G+C

This is the first most molecular method used as a tool to characterize microbes particularly for taxonomic purpose. Mol% G+C can be determined by thermal denaturation of DNA. This value ranges from 25 to 75 % depending upon the species of bacteria and is constant for a particular organism. It has been estimated that closely related organisms have almost similar mole% G+C profiles, and taxonomically related groups only differ between 3 and 5 %. This method is quantitative and not influenced by PCR biases and includes all DNA extracted. However, similar base composition is not a confirmation of relationship. If there is a difference in base composition, this is a worthy evidence of missing relationship. Moreover, it requires comparatively larger quantities of DNA (Clegg et al. 2000).

1.6.3 DNA Reassociation

This method and its kinetics imitate the variability of sequences by measuring the genetic complexity leading to diversity of microbes present in the environment (Torsvik et al. 1996). During this method, total DNA is extracted from environmental samples and further purified, denatured, and allowed to reanneal. The rate of hybridization or reassociation will depend on the similarity of sequences present. As the complexity or diversity of DNA sequences increases, the rate at which DNA reassociates will decrease (Theron and Cloete 2000). This method is influenced by several factors such as concentration of DNA product (Co) and time of incubation (t), usually described as the half association value, Cot1/2 (the time needed for half of the DNA to reassociate).

1.6.4 Nucleic Acid Hybridization

Hybridization method such as dot blot method is an important tool used in molecular bacteriology for both qualitative and quantitative analysis of DNA or RNA by using specific oligonucleotide probes which are designed from known sequences ranging in specificity from domain to species and are tagged with markers at the 5′-end (Goris et al. 2007). One of the most popular DNA hybridization methods is

FISH (fluorescent in situ hybridization). By using this method, spatial distribution of microbial communities in different environments such as biofilms can be determined. However, lack of sensitivity of hybridization of nucleic acids extracted directly from environmental samples is the most notable limitation of this method because of the fact that if sequences are not present in high copy number as in the case of dominant species, probability of detection is comparatively low.

1.6.5 Restriction Fragment Length Polymorphism

Restriction fragment length polymorphism (RFLP) relies on DNA polymorphisms and is proved to be a useful tool to study different microbial communities (Moyer et al. 1996). It is a simple and powerful tool for the identification of bacterial strains at and below species level and also for detecting structural changes in microbial communities. In this method, electrophoresed digests are blotted from agarose gels onto nitrocellulose or nylon membranes and hybridized with appropriate probes prepared from cloned DNA segments of related organisms. This method has been found to be very useful particularly in combination with DNA–DNA hybridization and enzyme electrophoresis for the differentiation of closely related strains and also during determination of intraspecies variation. However, it is to be noted that a similar banding pattern does not necessarily indicate a very close relationship between the organisms compared.

1.6.6 Terminal Restriction Fragment Length Polymorphism

This technique is an extension of the RFLP and overcomes some of its limitation. It provides an alternate method for rapid analysis of microbial community diversity in various environments (Thies 2007). This method has the same principle as RFLP except that one PCR primer is labeled with a fluorescent dye, such as TET (4, 7, 2′, 7′-tetrachloro-6-carboxyfluorescein) or 6-FAM (phosphoramidite fluorochrome 5-carboxyfluorescein). PCR is performed on sample DNA using universal l6S rDNA primers, one of which is fluorescently labeled. Fluorescently labeled terminal RFLP (FLT-RFLP) patterns can then be created by digestion of labeled amplicons using restriction enzymes. Fragments are then separated by gel electrophoresis using an automated sequence analyzer. Each unique fragment length can be counted as an operational taxonomic unit (OTU), and the frequency of each OTU can be calculated. The banding pattern can be used to measure species richness and evenness as well as similarities between samples.

However, this method may underestimate true diversity because only numerically dominant species are detected due to the large quantity of available template DNA (Liu et al. 1997). Incomplete digestion by restriction enzymes could also lead to an overestimation of diversity (Osborn et al. 2000). It has also been observed that

none of the presently available universal primers can amplify all sequences from eukaryote, bacterial, and archaeal domains. Additionally, these primers are based on existing 16S rRNA, 18S rRNA, or internal transcribed spacer (ITS) databases, which until recently contained mainly sequences from culturable microorganisms and therefore may not be representative of the true microbial diversity in a sample (Rudi et al. 2007). In addition, different enzymes will produce different community fingerprints. T-RFLP has also been thought to be an excellent tool to compare the relationship between different samples (Dunbar et al. 2000). T-RFLP has been used to measure spatial and temporal changes in bacterial communities (Lukow et al. 2000), to study complex bacterial communities (Moeseneder et al. 1999), to detect and monitor populations (Tiedje et al. 1999), and to assess the diversity of arbuscular mycorrhizal fungi (AMF) in the rhizosphere of *Viola calaminaria* in a metal-contaminated soil (Tonin et al. 2001).

1.6.7 Ribosomal Intergenic Spacer Analysis/Automated Ribosomal Intergenic Spacer Analysis/Amplified Ribosomal DNA Restriction Analysis

These methods are similar in principle to RFLP and T-RFLP and provide ribosomal-based fingerprinting of the microbial community. In RISA and ARISA, the intergenic spacer (IGS) region between the 16S and 23S ribosomal subunits is amplified by PCR, denatured and separated on a polyacrylamide gel under denaturing conditions. This region may encode tRNAs and is useful for differentiating between bacterial strains and closely related species because of heterogeneity of the IGS length and sequence. Sequence polymorphisms are detected by silver staining during this method. In ARISA, fluorescently labeled forward primer is detected automatically. Both RISA and ARISA method can deduce highly reproducible bacterial community profiles. Limitations of RISA include requirement of large quantities of DNA, relatively longer time requirement, insensitivity of silver staining in some cases, and low resolution (Fisher and Triplett 1999). ARISA has increased sensitivity than RISA and is less time-consuming, but traditional limitations of PCR also apply for ARISA (Fisher and Triplett 1999). RISA has been used to compare microbial diversity in soil, in the rhizosphere of plants (Borneman and Triplett 1997).

1.7 DNA Microarrays

This method is highly valuable for carrying out bacterial studies with high specificity because of the fact that a single array can contain thousands of DNA sequences (De Santis et al. 2007). Specific target genes coding for enzymes such

as nitrogenase, nitrate reductase, naphthalene dioxygenase, etc., can be used in microarray to elucidate functional diversity information of a community. The sample of environmental "standards" (DNA fragments with less than 70 % hybridization) representing different species likely to be found in any environment can also be used in microarray (Greene and Voordouw 2003). Reverse sample genome probing (RSGP) is the alternative method which uses genome microarrays to analyze microbial community composition of the most dominant culturable species in an environment as follows:

(a) Isolation of genomic DNA from pure cultures
(b) Cross-hybridization testing to obtain DNA fragments with less than 70 % cross-hybridization (DNA fragments with greater than 70 % cross-hybridization are considered to be of the same species)
(c) Preparation of genome arrays onto a solid support
(d) Random labeling of a defined mixture of total community DNA and internal standard

These methods may have thousands of target gene sequences but only detects the most abundant species. Furthermore, the bacterial species need to be cultured, but in principle cloned DNA fragments of unculturables could also be used. To avoid the cross-hybridization, enriched cultures should be used. It has also been observed experimentally that using genes or DNA fragments instead of genomes on the microarray offers the advantages of eliminating the need to keep cultures of live organisms, as genes can be cloned into plasmids or PCR can continuously be used to amplify the DNA fragments (Gentry et al. 2006). In addition, fragments would increase the specificity of hybridization over the use of genomes, and functional genes in the community could be assessed (Greene and Voordouw 2003).

1.8 Denaturant Gradient Gel Electrophoresis/ Temperature Gradient Gel Electrophoresis

In DGGE or TGGE, DNA fragments of the same length but with different base-pair sequences can be separated. DNA is extracted from natural samples and amplified using PCR with universal primers targeting part of the 16S or 18S rRNA sequences. The separation is based on the difference in mobility of partially melted DNA molecules in acrylamide gels containing a linear gradient of DNA denaturants (such as urea and formamide). Sequence variation within the DNA fragments causes a difference in melting behavior and hence in separation in denaturing gradient gels. The melting of the products occurs in different melting domains, which are stretches of nucleotides with identical melting temperatures (Muhling et al. 2008). Sequence variations in different fragments will terminate migration at different positions in the gel according to the concentration of the denaturant.

Theoretically, DNA sequences having a difference in only one base-pair can be separated by DGGE (Miller et al. 1999).

TGGE employs the same principle as DGGE with only difference is that gradient is temperature rather than chemical denaturants. Both methods are reliable, reproducible, rapid, and not much expensive. Multiple samples can be analyzed simultaneously, and tracking changes in microbial population in response to any stimuli or adversity is also possible by these methods. However, PCR biases, laborious sample handling, and variable DNA extraction efficiency are some of the limitations.

1.9 Single Strand Conformation Polymorphism

Single-strand conformation polymorphism (SSCP) relies on electrophoretic separation based on differences in DNA sequences and allows differentiation of DNA molecules having the same length but different nucleotide sequences and was originally developed to detect known or novel polymorphisms or point mutations in DNA (Peters et al. 2000). During this method, single-stranded DNA separation on polyacrylamide gel occurs due to differences in mobility resulting from their folded secondary structure, i.e., heteroduplex. This method reproduces an insight of the genetic diversity in a microbial community. However, some ss-DNA can exist in more than one stable conformation, and as a result, the same DNA sequence can produce multiple bands on the gel (Tiedje et al. 1999). However, it does not require a GC clamp or the construction of gradient gels and has been used to study bacterial or fungal community diversity, rhizosphere communities (Stach et al. 2001), and AMF species in roots (Kjoller and Rosendahl 2000).

1.10 Next-Generation Sequencing

Next-generation sequencing (NGS) methods that involve high-throughput sequencing such as pyrosequencing and illumina-based sequencing are modern tools with the possibility to discover new groups of microorganism(s) in complex environmental systems without cultivating them, and these real-time sequencing techniques are shedding light into the complexities of microbial populations significantly (Bartram et al. 2011). By using this method, highly complex microbiota compositions can be revealed with greater accuracy, and it is also possible to link microbial community diversity with niche function. It and can effectively provide deep insights into complex microbial communities in ecological niches (Fakruddin and Mannan 2013). Pyrosequencing is developed by Roche 454 Life Science, which can generate a huge amount of DNA reads. Recently, it has been successfully applied to study complex microbial environments such as the human gastrointestinal tract, soil, wastewater, and marine sediments. Besides

eliminating the use of cloning vectors and library construction, and their associated biases, NGS can also read through secondary structures and produce vast amount of sequences of up to 100 Mb per run (Royo et al. 2007). Furthermore, various bioinformatics tools such as the Newbler Assembler and RDP Pyrosequencing Pipeline have used to process and analyze NGS raw data in silico to rapidly determine the complex microbial composition and structure in environmental samples (Van den Bogert et al. 2011).

1.11 Metagenomics

It is defined as the functional and sequence-based analysis of the collective microbial genomes that are contained in an environmental sample (Zeyaullah et al. 2009). The collective genome, i.e., *metagenome* or *microbiome* of coexisting microbes, is randomly sampled from the environment and subsequently sequenced (Ghazanfar et al. 2010). Metagenomics give an illustrative view of the genetic diversity, species composition, evolution, and interactions with the environment of natural microbial communities (Simon and Daniel 2011).

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References

Arbeit RD (1995) Laboratory procedures for the epidemiologic analysis of microorganisms. In: Murray PR, Baron EJ, Pfaller MA, Tenover FC, Yolken RH (eds) Manual of clinical microbiology, 6th edn. American Society of Microbiology, Washington, DC, pp 190–208

Banowetz GM, Whittaker GW, Dierksen KP, Azevedo MD, Kennedy AC, Griffith SM, Steiner JJ (2006) Fatty acid methyl ester analysis to identify sources of soil in surface water. J Environ Qual 3:133–140

Bartram AK, Lynch MDJ, Stearns JC, Moreno-Hagelsieb G, Neufeld JD (2011) Generation of multimillion-sequence 16S rRNA gene libraries from complex microbial communities by assembling paired-end illumina reads. Appl Environ Microbiol 77:3846–3852

Borneman J, Triplett EC (1997) Molecular microbial diversity in soils from eastern Amazonia: evidence for unusual microorganisms and microbial population shifts associated with deforestation. Appl Environ Microbiol 63:2647–2653

Clegg CD, Ritz K, Griffiths BS (2000) %G+C profiling and cross hybridisation of microbial DNA reveals great variation in below-ground community structure in UK upland grasslands. Appl Soil Ecol 14:125–134

De Santis TZ, Brodie EL, Moberg JP, Zubieta IX, Piceno YM, Andersen GL (2007) High-density universal 16S rRNA microarray analysis reveals broader diversity than typical clone library when sampling the environment. Microb Ecol 53:371–383

Dokic L, Savić M, Narančić T, Vasiljević B (2010) Metagenomic analysis of soil microbial communities. Arch Biol Sci Belgrade 62:559–564

Dunbar J, Ticknor LO, Kuske CR (2000) Assessment of microbial diversity in four southwestern United States soils by 16S rRNA gene terminal restriction fragment analysis. Appl Environ Microbiol 66:2943–2950

Fakruddin M, Mannan MKSB (2013) Methods for analyzing diversity of microbial communities in natural environments. Ceylon J Sci (Bio Sci) 42:19–33

Fisher MM, Triplett EW (1999) Automated approach for ribosomal intergenic spacer analysis of microbial diversity and its application to freshwater bacterial communities. Appl Environ Microbiol 65:4630–4636

Garland JL, Mills AL (1991) Classification and characterization of heterotrophic microbial communities on the basis of patterns of community-level-solecarbon-source utilization. Appl Environ Microbiol 57:2351–2359

Gentry TJ, Wickham GS, Schadt CW, He Z, Zhou J (2006) Microarray applications in microbial ecology research. Microb Ecol 52:159–175

Ghazanfar S, Azim A, Ghazanfar MA, Anjum MI, Begum I (2010) Metagenomics and its application in soil microbial community studies: biotechnological prospects. J Anim Plant Sci 6:611–622

Giovannoni SJ, Britschgi TB, Moyer CL, Field KG (1990) Genetic diversity in Sargasso Sea Bacterioplankton. Nature 345:60–62

Glynn B, Lahiff S, Wernecke M, Barry T, Smith TJ, Maher M (2006) Current and emerging molecular diagnostic technologies applicable to bacterial food safety. Int J Dairy Technol 59:126–139

Goris J, Konstantinidis KT, Klappenbach JA, Coenye T, Vandamme P, Tiedje JM (2007) DNA–DNA hybridization values and their relationship to whole-genome sequence similarities. Int J Syst Evol Microbiol 57:81–91

Gray PHH (1926) A method of staining bacterial flagella. J Bacteriol 12:273–274

Greene EA, Voordouw G (2003) Analysis of environmental microbial communities by reverse sample genome probing. J Microbiol Methods 53:211–219

Hagens S, Loessner MJ (2007) Application of bacteriophages for detection and control of foodborne pathogens. Appl Microbiol Biotechnol 76:513–519

Holst-Jensen A, Rønning SB, Løvseth A, Berdal KG (2003) PCR technology for screening and quantification of genetically modified organisms (GMOs). Anal Bioanal Chem 375:985–993

Kell DB, Kaprelyants AS, Weichart DH, Harwood CR, Barer MR (1998) Viability and activity in readily culturable bacteria: a review and discussion of the practical issues. Antonie Van Leeuwenhoek 73:169–187

Kjoller R, Rosendahl S (2000) Detection of arbuscular mycorrhizal fungi (Glomales) in roots by nested PCR and SSCP (single stranded conformation polymorphism). Plant Soil 226:189–196

Kretzer JW, Lehmann R, Schmelcher M, Banz M, Kim K-P, Korn C, Loessner MJ (2007) Use of high-affinity cell wall-binding domains of bacteriophage endolysins for immobilization and separation of bacterial cells. Appl Environ Microbiol 73:1992–2000

Leifson E (1930) Staining, shape and arrangement of bacterial flagella. J Bacteriol 62:377–389

Liu WT, Marsh TL, Cheng H, Forney LJ (1997) Characterization of microbial diversity by determining terminal restriction fragment length polymorphisms of genes encoding 16S rRNA. Appl Environ Microbiol 63:4516–4522

Lukow T, Dunfield PF, Liesack W (2000) Use of the T-RFLP technique to assess spatial and temporal changes in the bacterial community structure within an agricultural soil planted with transgenic and non-transgenic potato plants. FEMS Microbiol Ecol 32:241–247

Lyon H (1991) Theory and strategy in histochemistry. Springer, Berlin

Malorny B, Tassios PT, Rådström P, Cook N, Wagner M, Hoorfar J (2003) Standardization of diagnostic PCR for the detection of foodborne pathogens. Int J Food Microbiol 83:39–48

Miller KM, Ming TJ, Schulze AD, Withler RE (1999) Denaturing Gradient Gel Electrophoresis (DGGE): a rapid and sensitive technique to screen nucleotide sequence variation in populations. BioTechniques 27:1016–1030

Minsky M (1988) Memoir on inventing the confocal microscope. Scanning 10:128–138

Moeseneder MM, Arrieta JM, Muyzer G, Winter C, Herndl GJ (1999) Optimization of terminal-restriction fragment length polymorphism analysis for complex marine bacterioplankton communities and comparison with denaturing gradient gel electrophoresis. Appl Environ Microbiol 65:3518–3525

Monis PT, Giglio S (2006) Nucleic acid amplification-based technique for pathogen detection and identification. Infect Genet Evol 6:2–12

Moyer CL, Tiedje JM, Dobbs FC, Karl DM (1996) A computer-simulated restriction fragment length polymorphism analysis of Bacterial Small-subunit rRNA genes: efficacy of selected tetraneric restriction enzymes for studies of microbial diversity in Nature. Appl Environ Microbiol 62:2501–2507

Muhling M, Woolven-Allen J, Murrell JC, Joint I (2008) Improved group-specific PCR primers for denaturing gradient gel electrophoresis analysis of the genetic diversity of complex microbial communities. ISME J 2:379–392

Osborn AM, Moore ERB, Timmis KN (2000) An evaluation of terminal-restriction fragment length polymorphisms (TRFLP) analysis for the study of microbial community structure and dynamics. Environ Microbiol 2:39–50

Pelczar JM, Chan ECS, Krieg R (1998) Noel microbiology, 5th edn. Tata McGraw-Hill Publishing Company Limited, New Delhi

Peters S, Koschinsky S, Schwieger F, Tebbe CC (2000) Succession of microbial communities during hot composting as detected by PCR-single-strand-conformation polymorphism based genetic profiles of small-subunit rRNA genes. Appl Environ Microbiol 66:930–936

Prescott ML, Harley PJ, Klein AD (2005) Microbiology, 6th edn. McGraw-Hill Higher Education, New York

Rodríguez-Lázaro D, Lombard B, Smith H, Rzezutka A, D'Agastino M, Helmuth R, Schroeter A, Malorny B, Miko A, Guerra B, Davison J, Kobilinsky A, Hernández M, Berhteau Y, Cook N (2007) Trends in analytical methodology in food safety and quality: monitoring microorganisms and genetically modified organisms. Trends Food Sci Technol 18:306–319

Royo JL, Hidalgo M, Ruiz A (2007) Pyrosequencing protocol using a universal biotinylated primer for mutation detection and SNP genotyping. Nat Protoc 2:1734–1739

Rudi K, Zimonja M, Trosvik P, Næs T (2007) Use of multivariate statistics for 16S rRNA gene analysis of microbial communities. Int J Food Microbiol 120:95–99

Saiki RK, Scharf S, Faloona F, Mullis KB, Horn GT, Erlich HA, Arnheim N (1985) Enzymatic amplification of ß-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. Science 230:1350–1354

Simon C, Daniel R (2011) Metagenomic analyses: past and future trends. Appl Environ Microbiol 77:1153–1161

Stach JEM, Bathe S, Clapp JP, Burns RG (2001) PCR-SSCP comparison of 16S rDNA sequence diversity in soil DNA obtained using different isolation and purification methods. FEMS Microbiol Ecol 36:139–151

Tabacchioni S, Chiarini L, Bevivino A, Cantale C, Dalmastri C (2000) Bias caused by using different isolation media for assessing the genetic diversity of a natural microbial population. Microb Ecol 40:169–176

Theron J, Cloete TE (2000) Molecular techniques for determining microbial diversity and community structure in natural environments. Crit Rev Microbiol 26:37–57

Thies JE (2007) Soil microbial community analysis using terminal restriction fragment length polymorphisms. Soil Sci Soc Am J 71:579–591

Tiedje JM, Asuming-Brempong S, Nusslein K, Marsh TL, Flynn SJ (1999) Opening the black box of soil microbial diversity. Appl Soil Ecol 13:109–122

Tonin C, Vandenkoornhuyse P, Joner EJ, Straczek J, Leyval C (2001) Assessment of arbuscular mycorrhizal fungi diversity in the rhizosphere of *Violoacalaminaria* and effect of these fungi on heavy metal uptake by clover. Mycorrhiza 10:161–168

Torsvik V, Sorheim R, Goksoyr J (1996) Total bacterial diversity in soil and sediment communities—a review. J Ind Microbiol 17:170–178

Touron A, Berthe T, Pawlak B, Petit F (2005) Detection of Salmonella in environmental water and sediment by a nested-multiplex polymerase chain reaction assay. Res Microbiol 156:541–553

Towner KJ, Cockayne A (1993) Molecular methods for microbial identification and typing, 1st edn. Chapman & Hall, London, pp. 1–202

Van den Bogert B, de Vos WM, Zoetendal EG, Kleerebezem M (2011) Microarray analysis and barcoded pyrosequencing provide consistent microbial profiles depending on the source of human intestinal samples. Appl Environ Microbiol 77:2071–2080

Vandamme P, Pot B, Gillis M, de Vos P, Kersters K, Swings J (1996) Polyphasic taxonomy, a consensus approach to bacterial systematics. Microbiol Rev 60:407–438

Voutou B, Stefanaki E-C (2008) Electron microscopy: the basics. Physics of advanced materials winter school. Aristotle University of Thessaloniki, Greece

Woeste S, Demchick P (1991) New version of the negative stain. Appl Environ Microbiol 57:1858–1859

YashRoy RC (1990) Lamellar dispersion and phase separation of chloroplast membrane lipids by negative staining electron microscopy. J Biosci 15:93–98

Zernike F (1942) Phase contrast, a new method for the microscope observation of transparent objects. Physica 9:686–698

Zeyaullah M, Kamli MR, Islam B, Atif M, Benkhayal FA, Nehal M, Rizvi MA, Ali A (2009) Metagenomics—an advanced approach for noncultivable micro-organisms. Biotechnol Mol Bio Rev 4:49–54

Zhao L, Ma T, Gao M, Gao P, Cao M, Zhu X, Li G (2012) Characterization of microbial diversity and community in water flooding oil reservoirs in China. World J Microbiol Biotechnol 28:3039–3052

Chapter 2
Novel Approaches to Identify and Characterise Microorganisms in Food Industry

Gamze Nur Kara, Mustafa Guzel, and Bulent Kabak

Abstract As far as food industries are concerned, two utmost important and determining aspects are safety and quality; therefore, it is highly important to detect, identify, characterise and quantify microorganisms in foodstuffs with greater accuracy. Modern technologies such as culture-independent molecular methods have solved existing problems associated with traditional culturing techniques. However, each molecular technique offers some advantages and limitations, and it is not an easy approach to choose appropriate molecular method that may depend on the objective studies and several variables such as time to obtain results, cost, reproducibility, etc. Studies on the development and optimisation of molecular techniques are of great importance in the detection of biological hazards in foodstuffs.

2.1 Introduction

Food products can be contaminated with food-spoilage microorganisms and food pathogens at various stages in the food chain. Microorganisms can be present in raw products (agricultural or animal) prior to harvesting, or they may contaminate during slaughter or processing, by the addition of contaminated food ingredients or processing aids, from the factory environment or by cross-contamination from other contaminated foods or from food handlers. Food-spoilage microorganisms such as bacteria and fungi (yeast and mould) spoil food by growing in it and producing substances that change the physical and chemical properties of the food, including colour, texture and odour of the food (Ceuppens et al. 2014). Food-borne pathogens can enter the body by consuming contaminated foodstuffs or drinking water and/or through contact with infected animals and cause various

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diseases in humans, from mild symptoms to long-lasting disability and death. Food-borne zoonotic diseases are a significant widespread global public health threat. In the European Union (EU), over 320,000 human cases are reported each year, of which 200,000 are caused by *Campylobacter jejuni* and 100,000 by *Salmonella* spp. However, the real number is likely to be much higher. Moreover, food-borne pathogens are estimated to cause nine million illnesses and about 1000 deaths each year in the United States (Scallan et al. 2011). It has been estimated that the annual cost of health losses linked to food-borne diseases is more than $77 billion (Scharff 2012). The most common bacteria that cause food-borne diseases are *Campylobacter jejuni*, *Salmonella* spp., *Listeria monocytogenes*, pathogenic *Escherichia coli*, *Yersinia enterocolitica*, *Staphylococcus aureus*, *Clostridium perfringens*, *Clostridium botulinum* and *Bacillus cereus*.

In recent years, food safety and food quality have been the issues increasingly dominating public and political debate (Saron 2007). Therefore, monitoring of microbial flora of foodstuffs is important to prevent health hazards and economic losses. Conventional microbiological methods for testing foodstuffs are based on growth in culture media, followed by isolation/enumeration and biological and serological identification. Due to the fact that conventional methods are labour-intensive and time-consuming, requiring from days to weeks to get results, and may lead to uncertain identification or misidentification, more accurate, rapid and effective new approaches based on the application of immunological (such as enzyme-linked immunosorbent assay) or molecular techniques have been developed in the detection of microorganisms in the last years (Fratamico and Bayles 2005). The most recent molecular approaches to detect and identify food-borne microorganisms have been discussed in this chapter.

2.2 Molecular Approaches to Detect and Identify Food-Borne Microorganisms

The aim of molecular biology is to study the genome, transcriptome and proteome. Within the last three decades, there have been considerable advances in the development and use of molecular techniques for the detection of microorganisms in foodstuffs as a result of the increasing demand for rapid results. These are normally based on detecting specific nucleic acid (DNA or RNA) target sequences using amplification processes and also identifying protein expression under specific conditions (Ceuppens et al. 2014).

2.2.1 Nucleic Acid-Based Methods

2.2.1.1 Nucleic Acid Hybridisation

The identification of bacteria by DNA probe hybridisation methods is based on the presence or absence of particular genes. A different kind of technologies based on the nucleic acid hybridisation, such as Western blot, Northern blot, Southern blot, dot blot, colony hybridisation, colorimetric hybridisation, etc., have been developed and successfully applied to the pathogen detection. However, a major drawback of the hybridisation assays is their lack of sensitivity, which limits the use of these analyses to populations of cells or genes occurring in relatively high numbers in samples. Thus, these assays are currently mainly used for culture confirmation rather than direct detection and identification (Fusco and Quero 2012).

Among all the hybridisation assays, fluorescence in situ hybridisation (FISH), developed in the 1980s, is given a particular attention. In this method, small DNA strands [15–30 base pairs (bp)], which are labelled with fluorescent, are used as probe. Following the denaturation step of target double-stranded DNA (dsDNA), this probe binds as complementary strand to specific part of single-stranded DNA (ssDNA) for hybridisation. After hybridisation, pathogen bacteria cells can be detected with fluorescence microscopy or flow cytometry without the need for cultivation (Pernthaler et al. 2001).

FISH assays have been used to detect at genus and species level of various food-borne pathogens such as *Staphylococcus* spp., *Listeria* spp. (Fuchizawa et al. 2008), *Campylobacter* spp., *Salmonella* spp. and *Escherichia coli* (Stender et al. 2001) isolated from different foodstuffs. For instance, 16S rRNA FISH method has been used for detecting in situ of domain microorganisms within the cheese matrices (Ercolini et al. 2003). In another study, Fang et al. (2003) identified *Salmonella* species in food with the use of FISH with 23S rRNA-targeted oligonucleotide probes, which are Sal-1 and Sal-3. Similarly, Bisha and Brehm-Stecher (2010) used FISH combined with adhesive taped-based sampling method to determine *Salmonella* spp. on fresh vegetable surfaces.

2.2.1.2 Polymerase Chain Reaction

The polymerase chain reaction (PCR) is a powerful technique, which is used to amplify, or increase the amount of, a specific DNA target sequence (Hayashi 2012). In general, PCR process consists of at least 35–40 cycles and each cycle comprises three different temperature-step processes: denaturation, annealing and elongation. At denaturation step, dsDNA is separated into two ssDNA (melting) through the breaking of the hydrogen bonding between the base pairs by application of high temperature (92–96 °C) in the reaction tube. Temperature at which half of the DNA molecules become single-stranded is named as melting temperature (T_m). The second step is the annealing of the DNA primers to form the specific

complementary sequences to the target ssDNA molecules. These primers are short fragments of DNA that match up to the forming ends of the new DNA sequence of interest. This phase is performed at 30–65 °C. The final step in cycling is the elongation of the DNA, which involves binding of the deoxynucleotides (dNTPs) from the reaction mixture to the complementary target sequence with DNA polymerase enzyme (generally *Taq* polymerase) catalysing at 72–75 °C. Following the finished cycle, the dsDNAs are converted to ssDNAs by heating again and whole PCR process begins (Levin 2009).

The PCR and other rapid methods are often inhibited by food matrix components; thus, there is a need to separate and concentrate microorganisms or to purify microbial DNA from food samples or enrichments prior to detection. When performing the PCR, sample preparation is necessary to remove inhibitory substances such as complex polysaccharides, lipids and proteins that may reduce amplification efficiency, to increase the concentration of the target organism/DNA and to produce a homogeneous sample ensuring reproducibility of the assay. The methods that have been used for this purpose include aqueous polymer two-phase systems, immune-magnetic separation, centrifugation or filtration. Moreover, many DNA extraction kits and reagents are commercially available (Fratamico and Bayles 2005).

There are many PCR-based protocols that are used for detecting the desirable and undesirable microorganisms in food samples. Some of the most common PCR variants are random amplification of polymorphic DNA (RAPD)-PCR, real-time PCR (RTi-PCR), multiplex PCR (MPCR), PCR-restriction fragment length polymorphism (RFLP), DNA sequencing and multilocus sequence typing (MLST). The advantages and limitations of PCR-based methods have been summarised in Table 2.1.

RAPD-PCR

RAPD-PCR assay is based on the use of short random sequence primers (nine or ten bases in length), which can be applied to any species without information of nucleotide sequence. Primers hybridise chromosomal DNA sequences with a sufficient affinity at low annealing temperatures (Giraffa and Carminati 2008; Kumar and Gurusubramanian 2011). Amplicons exhibit strain-specific patterns of bands by separation with agarose gel electrophoresis due to the fact that the number and location of random primer sites vary for different strains of yeast and bacterial species (Bardakci 2001). This method has been reported to be profitable, simple and non-time-consuming. RAPD-PCR can be performed both on purified and untreated DNA (Giraffa and Carminati 2008).

The RAPD-PCR method has been reported to be useful for establishing the persistence of specific strains of lactic acid bacteria (LAB) (Plengvidhya et al. 2004; Seseña et al. 2005) and yeasts (Zahavi et al. 2015) in food products and also genetic typing of various food-borne pathogens (Keeratipibul and Techaruwichit 2012). LAB plays an essential role in some fermented products by decreasing food

Table 2.1 Advantages and limitations PCR-based methods

Method	Advantages	Limitations
RAPD	Simple Non-time-consuming Low expense It can be used on any DNA sample Target genome information is not required	Purified and high molecular weight genome template is essential Low reproducibility
RTi-PCR	Culture independent Sensitive Reliable Fast Easy to perform Not require post-PCR analysis	High equipment cost Requires high technological support for setting
MPCR	Cheap Time saving High sensitive High throughput Less input material	Difficult Primer dimers Sometimes results in non-specific amplifications
DNA sequencing	High discrimination level Accurate and fast results Sequencing reactions are directly obtained from PCR products	Require post-PCR analysis More expense Lower throughput
MLST	Portable Robust Easy to perform	Expensive Not all organisms are suited for MLST analysis
RFLP	Culture independent Easy to perform Inexpensive Simple Powerful	Need long time from start to completion Large amount of DNA required Some enzymes are costly Different enzyme composition needed

pH as a result of producing lactic acid and producing aroma compounds (Centeno and Carballo 2014). Yeasts are also abundant and essential microorganisms in some fermented foods and beverages. However, they are also spoilage microorganisms in some food products (Arroyo-López et al. 2008). In a previous study, Lucena-Padrós et al. (2014) identified both yeast and LAB genotype isolated from Spanish-style fermented green table olives by using RAPD-PCR method. In another study, *Klebsiella pneumoniae* as an entero-invasive food-borne pathogen was isolated from street foods in Malaysia and characterised by RAPD-PCR fingerprinting (Haryani et al. 2007).

RTi-PCR

RTi-PCR is one of the PCR-based techniques that is increasingly applied for detection and molecular quantification of microorganisms in food products. This technique is also called as “homogenous PCR”, “kinetic PCR” or “qPCR” (Mackay

et al. 2007). The basic principle of the system is monitoring the amplification of the target DNA in real time by the employment of fluorescent technology (Stjepanovic et al. 2011). Many alternative instruments and fluorescent probe systems have been developed to use in this technique. Two common methods are used to detect amplicon: DNA-binding dyes such as ethidium bromide (EtBr) and SYBR Green I or specifically bind probes such as TaqMan® and FRET (fluorescence resonance energy transfer) probes (Foley and Grant 2007). SYBR Green I, one of the most frequently used fluorescent DNA-binding dye in RTi-PCR application, binds all dsDNA, and detection is monitored by measuring the increase in fluorescence throughout the cycle. TaqMan® probe is also named as "double-dye oligonucleotide", "5′ nuclease probe" and "dual labelled probe". The TaqMan® probe contains a fluorescent dye on one end and a quencher (that suppresses fluorescence) on the other. If the target sequence is present during the PCR and the probe binds to the target DNA, then the probe is degraded, resulting in an increase in fluorescence. The instrument detects accumulation of PCR products by monitoring the increase in fluorescence. This method is culture independent, sensitive, reliable, fast and easy to perform. In RTi-PCR method, data is monitored throughout the PCR process in real time instead of looking at bands on a gel at the end of the reaction. Moreover, this system is avoiding cross-contamination due to close-tube system (Mackay et al. 2007).

Many studies have been performed to identify yeasts, moulds and pathogen bacteria isolated from various foodstuffs in recent years. For instance, Rodríguez et al. (2011) performed two RTi-PCR methods based on SYBR Green I and TaqMan to detect patulin-producing moulds. More recently, Rodriguez-Lazaro et al. (2014) detected *Salmonella* spp. in raw pork, poultry meat, salad and raw sheep milk by using RTi-PCR-based methods.

MPCR

MPCR is a variant of PCR in which several gene sequences belonging to the same bacteria or originating from the mixture of different microorganisms simultaneously amplified in the same reaction. This method was first described in 1988 and has been widely used for detection of LAB in dairy products (Giraffa and Carminati 2008), food-borne pathogens (Chen et al. 2012) and their toxins (Singh and Mustapha 2015). Primer setting is an important point in MPCR technique. It is necessary to design the primers longer than those used in conventional PCR. The MPCR technique is cheaper, quicker, high sensitive and easier to run when compared to conventional PCR. Additionally, two or more pathogens can be detected in one tube without any enrichment step (Yu et al. 2016). This is important because many foods such as fruits, vegetables, milk, dairy, poultry and meat products may contain more than one pathogen. Singh and Mustapha (2015) detected simultaneously eight serogroups of Shiga toxin (Stx) produced by *Escherichia coli* (STEC), O145, O121, O104, O157, O26, O45, O103 and O111 in food samples within less than 11 h. More recently, Yu et al. (2016) studied on commercial cold

dishes food such as meat, vegetable and soy products purchased randomly from supermarkets in China and used both conventional and MPCR methods for the identification and characterisation of 99 bacteria isolates. *Staphylococcus aureus*, *Listeria monocytogenes* and *Salmonella* spp. were identified with both conventional and MPCR methods. The MPCR showed a wide range of specificity although only one or two template combinations were used.

PCR-RFLP

PCR-RFLP is another popular genotyping technique that is based on DNA extraction, amplification of a specific gene sequence by PCR and digestion of amplicons into smaller fragments with specific endonucleases that have been isolated from different bacterial species. Digested fragments are separated by gel electrophoresis according to their size (Giraffa and Carminati 2008). Several variants of PCR-RFLP method such as amplified fragment length polymorphism (AFLP), terminal restriction fragment length polymorphism (T-RFLP), inverse PCR-based amplified restriction fragment length polymorphism (iFLP) and PCR combined with restriction fragment melting temperature (PCR-RFMT) have been also used for detection of food-borne microorganisms.

PCR-RFLP method has been used in many studies to identify microorganisms since it is a very rapid, simple, powerful and inexpensive technique. In an earlier study, Espinosa et al. (2002) identified *Saccharomyces cerevisiae*, *Hansenia sporauvarum*, *Kluyveromyces thermotolerans*, *Candida vinaria* and *Candida stellate* using PCR-RFLP method. They reported that after 5.8S-ITS rDNA region of extracted DNA was amplified by PCR, *Hinf*I, *Hae*III and in some cases *Dde*I, endonucleases were used for restriction. It has been concluded that PCR amplification of 5.8S-ITS rDNA may be a good method for fast identification of the yeasts involved in wine fermentation, yielding information without the need of cultivation or time-consuming plate isolation methods. In another study, yeasts were isolated from fermented orange juice and subjected to diversity analysis. The analysis was conducted using restriction fragment length polymorphism on the ITS region (including ITS1, 5.8S rRNA gene and ITS2), which was amplified using PCR with ITS1 and ITS4 primers. Restriction enzymes used in this research were *Hha1*, *HinfI* and *HaeIII* (Soka and Susanta 2010). Similarly, Pham et al. (2011) generated and tested a 5.8S internal transcribed spacer (ITS) RFLP library for brewery wild yeast contaminants as well as ale and lager brewing yeast strains. The efficacy of the technique was assessed by isolation of 59 wild yeasts from industrial fermentation vessels conditioning tanks and by matching their ITS amplicon sizes and RFLP profiles with those of the constructed library. More recently, Marty et al. (2012) performed PCR-RFLP method to monitor pathogenic, spoilage and technologically important microorganisms in 21 spontaneously fermented Swiss meat products. The authors showed that *Lactobacillus sakei* and *Lactobacillus curvatus* were dominant species in fermented meat products followed by *Staphylococcus* species such as *S. saprophyticus*, *S. gallinarum*, *S. capitis*, *S. sciuri* and *S. epidermidis*.

DNA Sequencing

DNA sequencing is another PCR-based molecular method in which the nucleotide bases along a DNA strand are determined. There are two different methods used in sequence analysis: chemical cleavage and dideoxy chain termination that is also named as Sanger sequencing method (França et al. 2002). The most frequently used one is Sanger sequencing which is based on enzymatic DNA synthesis. The protocol of this method starts with DNA extraction from pure cultures, followed by PCR amplification. Four different reaction mixes are prepared for sequence analysis. Each mixture includes template DNA, primer, four dNTPs (A, G, C, T) and only small amount of one of the dideoxynucleotides (ddNTP; either ddA, ddC, ddG or ddT). In sequencing reaction, primers that anneal to a single-stranded DNA template are elongated by DNA (Taq) polymerase. Fluorescent-labeled deoxynucleotides are introduced on at a time and the primer is extended in a template-dependent manner. In the same reaction, Taq polymerase adds to denatured DNA, fluorescent-labelled modified (dideoxy) nucleotides that terminate the formation of a new DNA strand as they encounter their complementary nucleotides in the target sequence. This results in DNA strands of variable length, which are separated on a gel electrophoreses and reflect the sequence being analysed. (Rapley and Whitehouse 2007). The obtained result is compared with reference sequences through the basic local alignment search tool (BLAST) or other databases.

Sequencing method has been extensively used for many years for characterisation and identification of yeast (Pereira et al. 2015) and food-borne bacteria (El-Hadedy and El-Nour 2012; Aydemir et al. 2015; Kurtzman 2015).

MLST

MLST is another nucleotide sequencing-based subtyping method that includes sequencing housekeeping genes in different loci around the chromosome of microorganisms. Approximately, 7–10 housekeeping genes are chosen for this purpose. This method was firstly used by Maiden et al. (1998) to identify pathogenic *Neisseria meningitidis* species. MLST includes several steps: DNA extraction, PCR application to housekeeping genes, sequencing of PCR products, comparison of each sequence fragment, MLST allele assignment and data analysis (Muñoz et al. 2014). This method is portable, robust and easy to perform. Moreover, housekeeping genes are sufficiently stable that makes this method to be ideal for global epidemiology. Different analysis software is available on MLST websites that makes it easy to identify several bacterial species.

Many food-borne pathogens like *Campylobacter jejuni*, *Clostridium botulinum*, *Clostridium perfringens*, *Escherichia coli*, *Listeria monocytogenes*, *Bacillus cereus*, *Staphylococcus aureus*, *Salmonella enterica*, *Vibrio parahaemolyticus*, *Enterococcus faecium* and *Enterobacter sakazakii* have been identified by using MLST (Muñoz et al. 2014; Killer et al. 2015).

MLST has been also used for identification of bacteria involved in food fermentation such as *Oenococcus oeni*, *Lactobacillus plantarum*, *Lactobacillus casei*, *Lactobacillus paracasei*, *Lactobacillus sanfranciscensis*, *Lactobacillus delbrueckii*, *Lactobacillus lactis*, *Pediococcus parvulus*, *Pediococcus damnosus* and *Saccharomyces cerevisiae* (Muñoz et al. 2014). In a study conducted by Dan et al. (2014), MLST protocol was used for identifying *Leuconostoc lactis* isolates obtained from traditional dairy products in China and Mongolia, and eight different target housekeeping genes (*groEL*, *carB*, *recA*, *pheS*, *murC*, *pyrG*, *rpoB* and *uvrC*) were determined. More recently, a total of ten housekeeping genes (*carB*, *clpX*, *dnaA*, *murC*, *murE*, *pepN*, *pepX*, *pyrG*, *recA* and *rpoB*) were sequenced for identifying *Streptococcus thermophilus* strains isolated from traditional dairy products in China and Mongolia (Yu et al. 2015).

2.2.1.3 Pulsed-Field Gel Electrophoresis

Pulsed-field gel electrophoresis (PFGE) method is based on bacteria characterisation by digesting DNA with restriction enzymes into suitable DNA sequences (Li et al. 2009). Since the size of obtained DNA fragments is big, they cannot be separated by standard agarose or acrylamide gel electrophoresis technique (Olive and Bean 1999). The method allows separating DNA fragments and visualising them with ethidium bromide. The obtained DNA fingerprint reflects the DNA sequence, and the result is compared with the existing database for bacteria identification (Birren and Lai 2012).

PFGE method has been reported to exhibit high discrimination level in food pathogen detection. Favier et al. (2013) aimed to determine the prevalence of *Salmonella* spp. in foods of animal origin sold at retail stores over the period of 2005–2011 in San Luis, Argentina. PFGE assay was used with the combination of *Xba*I DNA restriction to determine possible genetic relationships among the *Salmonella* isolates. In another study, Rivoal et al. (2013) characterised *Listeria monocytogenes* isolated from egg break plants by PFGE using *Apa*I and *Asc*I enzymes. Recently, Muñoz-Atienza et al. (2016) investigated the safety of 14 potential probiotic *Enterococcus faecium* strains with antimicrobial activity. In this study, the genetic relatedness of *Enterococci* was determined by PFGE, RAPD, enterobacterial repetitive intergenic consensus (ERIC-PCR) and restriction analysis of amplified *16S rDNA* (ARDRA). ERIC-PCR yielded the highest diversity, followed by RAPD and PFGE, while ARDRA achieved the lowest diversity.

2.2.1.4 Ribotyping

Ribotyping is similar to PFGE since DNA is cut into fragments by an endonuclease such as *EcoRI* (Li et al. 2009). Differently from PFGE, genomic DNA is cut into smaller fragments around the number of 300–500 and 1–30 kb in size. Obtained fragments can be separated according to their size by agarose gel electrophoresis. In

the following, separated fragments are transferred to a nylon membrane and hybridised with an appropriately labelled copy DNA (cDNA) probe derived from ribosomal RNA (rRNA). Ribotyping is highly reproducible and cheap, while this technique is time-consuming and requires considerable technical expertise (Stjepanovic et al. 2011).

Ribotyping is useful for taxonomical and long-term epidemiological studies. De Cesare et al. (2007) determined *Listeria monocytogenes* strains genetic diversity isolated from fresh and fermented sausages by automated *Pvu*II ribotyping and predicted the pathogenicity lineage of *L. monocytogenes* isolates determining their DuPont Identification Library Codes (DUP-IDs) by *EcoR*I ribotyping. In another work, Salustiano et al. (2009) observed that ribotyping was most discriminatory to detect variations between strains of *Bacillus cereus* isolated from milk and the post-pasteurisation equipment surfaces.

2.2.2 Proteomics

Proteomics are used to identify protein expression under specific conditions. In the post-genomic era, proteomics is valuable in comparison of subspecies as well as determination of cell functions (Feng and Liu 2012). Proteomics is also useful in secretomics. In recent years, secretome studies increased the overall understanding of pathogen physiology, surface colonisation and virulence. Secretome studies not only cover the secretion machineries but also the secreted proteins. Today, in proteomics studies of food-borne pathogens, gel-based and gel-free methods are the most commonly deployed methods.

Gel-based methods were first introduced in 1975 with two-dimensional gel electrophoresis (2DGE) (Klose 1975; O'Farrell 1975). Since then, this technique has been used in comparative proteomics as it allows to analysing of proteins in different conditions (e.g. growth stages, environmental stresses). Furthermore, it is possible to evaluate the whole proteome in a single sample with 2DGE. A breakthrough in 2DGE happened with the introduction of fluorescent labelling which enabled analysis of three samples in one gel. This technique is called two-dimensional fluorescent difference gel electrophoresis (2D-DIGE), and not only does it help to decrease the time consumption and labour intensity; it also increases the reproducibility by decreasing the gel consumption and increasing the sample size (Bonar et al. 2015). After the separation of proteins with 2DGE or 2D-DIGE, spots of interest are selected for fingerprinting by matrix-assisted laser desorption/ionisation time-of-flight mass spectrometer (MALDI-TOF). Then, proteins are identified with a database search. Despite the certain advantages such as detection of intact proteins and post-translational modifications, gel-based methods have limited detection power of small amount proteins and speed.

The second approach is based on liquid chromatography mass spectrometry (LC-MS). In this method, isolated proteins are digested with a site-specific protease such as trypsin, and the fragments are separated with LC (Domon and Aebersold

2010). After this separation, MS is used for identification. This method is also called as *shotgun proteomics* because of the resemblance of shotgun genomics, and it offers greater sensitivity and broader dynamic ranges (Marcotte 2007). LC-MS approach covers the proteome better than 2DGE and is becoming the main approach consequently (Feng and Liu 2012). MS-based approach can further be modified with labelling methods such as stable isotope labelling by amino acids in cell culture (SILAC), isotope-coded affinity tag (ICAT), isotope-coded protein labelling (ICPL), tandem mass tags (TMT) or isobaric tags for relative and absolute quantification (iTRAQ) (Zhu et al. 2010).

Development of labelling methods is a big step forward as they led the way of quantified proteomics. However, apart from TMT and iTRAQ (eight samples at once), labelling methods enable comparison of limited number of samples. As a result, the interest in label-free shotgun proteomics has been increasing. In principle, label-free methods have four major steps:

(a) Preparation (i.e. protein extraction, alkylation and digestion)
(b) Separation with LC
(c) Analysis with MS
(d) Data analysis (e.g. identification and quantification).

Quantification in label-free approach is based on peak intensity or spectral counting and requires specific software (Marcotte 2007; Zhu et al. 2010). Currently, there are a number of commercial and open-source software developed for this job.

In the past 10 years, proteomics studies of several food-borne pathogens (*Campylobacter jejuni*, *Salmonella* spp., *Escherichia coli, Listeria monocytogenes*, *Staphylococcus aureus*, etc.) have been evaluated mainly in terms of physiological state, inactivation mechanisms, biofilm formation and virulence. Some of the current proteomics studies on food-borne pathogens are summarised in Table 2.2.

C. jejuni is one of the leading causes of food-borne diseases in both the European Union and United States. Scott and Cordwell (2009) reviewed the proteomics studies of *C. jejuni* and stated that *C. jejuni* was a unique pathogen due to the ability of making post-translational modifications. Recently, Elmi et al. (2012) and Jang et al. (2014) identified 151 and 134 vesicular proteins, respectively. Vesicular proteins are secreted from outer membrane vesicles of gram (–) bacteria and have significant roles in survival and virulence.

Encheva et al. (2005, 2007) evaluated the proteomics of *Salmonella* serotypes Typhimurium, Pullorum, Enteritidis, Choleraesuis and Dublin. It has been reported that the protein maps of strains were highly similar with each other. However, Sun and Hahn (2012) compared three serovars, Typhimurium, Enteritidis and Gallinarum, and observed significant differences in the expression of virulence proteins. In another study, Adkins et al. (2006) compared the proteomes of a virulent (ATCC 14028) and an attenuated (LT2) *S.* Typhimurium strains in magnesium-depleted medium. Researchers identified 2121 proteins for LT2 and 2296 proteins for 14028. The results showed that 90 % of these proteins were overlapped. Different expression levels, however, were shown in propanediol utilisation operon, which is associated with virulence. Cho et al. (2015) determined

Table 2.2 Current proteomics studies on food-borne pathogens

Pathogen	Technique	Conditions	Finding	Reference
L. monocytogenes	MudPIT and Q-TOF LC/MS	Sublethal nisin conditions	Nine proteins upregulated including oxidative stress response and flagellar proteins	Miyamoto et al. (2015)
L. monocytogenes	2-DE and MALDI TOF/TOF MS	High salinity, low pH and low temperature	Oxidative stress, protein synthesis, cell wall and nucleotide metabolism pathways affected	He et al. (2015)
E. coli O157:H7	2-DE and MALDI QTOF MS/MS	HCl, lactic acid (LA) and low temperature	OmpA upregulated in HCl stress and low temperature, downregulated in LA stress	Huang et al. (2015)
S. Enteritidis	2-DE and MALDI TOF MS	Outer membrane proteins were mapped	OmpA and Dps were the most abundant proteins	Cho et al. (2015)
V. parahaemolyticus	2-DE and MALDI Q-TOF LC/MS	NaOCl, acidic and slightly acidic water	OmpK and OmpU upregulated, protein synthesis related, proteins downregulated	Chen et al. (2016)
C. sakazakii	2-DE and MS/MS	Virulent and attenuated strains were compared	Five membrane proteins upregulated in virulent strain	Ye et al. (2015)
C. jejuni	2-DE and nano-LC-LTQ-Orbitrap MS	Outer membrane vesicles were profiled	134 proteins were identified, bioinformatics analysis predicted, redox proteins were enriched	Jang et al. (2014)

the antigenic outer membrane proteins of *S.* Enteritidis strains 270 and 350. The study showed that OmpA, Dps (DNA starvation/stationary phase protection protein) and possibly OmpW are antigenic. Antibiotic resistance of *Salmonella serovars* has been also studied extensively (Hu et al. 2007; Karatzas et al. 2008; Pinto et al. 2010; Correia et al. 2014).

Due to the fact that Shiga toxin-producing *E. coli* (STEC) and especially *E. coli* 0157:H7 cause the highest number of food-borne outbreaks (CDC 2015) within the pathogenic *Escherichia coli* group, majority of the studies have been focused on this pathotype. Lippolis et al. (2009) compared the proteomic changes in *E. coli* O32:H37, a mastitis-causing strain, in milk and culture media. Among the nearly 1000 proteins reported in the study, hundreds of proteins including quorum sensing and mobility proteins were differently expressed in milk. The study showed that the physical behaviour and virulence of pathogens in food environment is very different compared to model systems. Zhou et al. (2010) compared the proteomes of enterohaemorrhagic *E.coli* (EHEC) O157:H7 and *enteropathogenic E. coli* (EPEC) O55:H7 and found that 118 proteins were expressed differently.

Huang et al. (2007) studied the effect of acid stress on an O157:H7 strain. Survival of the non-acid-adapted pathogen was 95.7 % in stationary phase, whereas the survival rate of the cells in exponential phase was only 0.9 % at pH 3.0 for 2 h. In addition, when cells were adapted to acidic conditions, secretion of Shiga toxin (Stx) was decreased greatly compared to non-adapted cells. Authors suggested that changes in membrane lipids due to acid adaptation might result in decreased toxin secretion. Kaur and Chakraborti (2010) evaluated the acid response of an enteroaggregative *E. coli* (EAEC) strain. In the absence of *rpoS*, a stress regulatory gene, cells were unable to grow at pH 4. It was concluded that the absence of *rpoS* leads to downregulation of acid-induced proteins. In addition to their metabolic functions, outer membrane proteins play a vital role in virulence of O157:H7. For example, OmpA is found to be essential in adherence of the pathogen to human brain cells (Shin et al. 2005). Recently, Huang et al. (2015) evaluated the effect of low temperature and chemical treatments on the proteome of an O157:H7 strain. When subjected to HCl, OmpA was upregulated by 2.38-fold, whereas lactic acid treatment was resulted in a downregulation by 1.9-fold. Similar observations were made for OmpX, which was upregulated by 1.6-fold and downregulated by 3.9-fold by HCl and lactic acid, respectively.

Many studies have been conducted on the secretion system of *L. monocytogenes*. Donaldson et al. (2009) compared global protein expression of two *L. monocytogenes* strains, EGD and F2365, during early stationary phase. 1754 proteins in EGD and 1427 proteins in F2365 were identified. Among those proteins, 1077 proteins were common in both strains. Significant differences were found in the expression of cell wall, flagellar, DNA repair and stress response proteins. In a similar study, Donaldson et al. (2011) compared the protein expression profiles of three *L. monocytogenes* strains, EGDe, F2365 and avirulent HCC23, isolated from murine macrophages. Although all strains expressed metabolic proteins for 3 h, avirulent strain, HCC23, was unable to cause prolonged infection. On the other hand, strains EGDe and F2365 proliferated in macrophage for 7 h. It has been suggested that prolonged proliferation was linked to DNA repair and stress response proteins. Huang et al. (2014) compared proteomic variations of *L. monocytogenes* with a clinical isolate and two food-borne isolates. It has been reported that clinical isolate showed 53.4 and 53.9 % similarity to dairy and seafood isolates, respectively. Furthermore, significant variations were determined in the expression of specific proteins such as cold shock proteins.

In another study, Agoston et al. (2009) evaluated the protein expression of *L. monocytogenes* under various heat conditions using two-dimensional gel electrophoresis coupled with matrix-assisted laser desorption/ionisation time-of-flight analysis. The cells were subjected to heat shock at 60 °C, and 18 proteins were expressed differently. When the heat shock prolonged to 9 min, 21 proteins including DnaN (stress protein), TcsA (a lipoprotein) and Gap (glyceraldehyde-3-phosphate-dehydrogenase) were expressed differently compared to control. More recently, protein expression of *L. monocytogenes* in the presence of nisin was studied by Miyamoto et al. (2015). It was found that nine proteins were upregulated and four were downregulated compared to control group. Highly expressed proteins

were associated with oxidative stress, flagellar movement and cell membrane production.

The proteome of *S. aureus* has been studied in terms of antibiotic resistance (Monteiro et al. 2012), secretome (Kusch and Engelmann 2014), biofilm formation (Resch et al. 2006), pathogenicity (Bonar et al. 2015) and extracellular proteins (Enany et al. 2014). Lee et al. (2009) determined that *S. aureus* membrane vesicles release biologically active BlaZ, a β-lactamase protein, which protects ampicillin-susceptible bacteria against ampicillin. Authors also discovered that the protective function of BlaZ was not observed after heat treatment of vesicles.

Proteomic changes in *Vibrio parahaemolyticus* under the effect of three chemical sanitisers, acidic electrolysed water, slightly acidic electrolysed water and sodium hypochlorite solution, were determined by Chen et al. (2016). The sanitisers affected cell membrane, protein synthesis and ATP biosynthesis pathways. It has been concluded that employment of proteomics is a valid approach to determine inactivation mechanisms.

2.3 Conclusions

Food safety and food quality are two important aspects in food industry. Thus, reliable paths to detect, identify, characterise and quantify microorganisms in foodstuffs are of great interest. Culture-independent molecular methods in food microbiology have solved existing problems associated with traditional culturing techniques. However, each molecular technique offers some advantages and limitations. It is not easy to choose appropriate molecular method and may depend on the objective studies and several variables such as time to obtain results, cost, reproducibility, etc. Studies on the development and optimisation of molecular techniques are of great importance in the detection of biological hazards in foodstuffs.

References

Adkins JN, Mottaz HM, Norbeck AD, Gustin JK, Rue J, Clauss TRW, Purvine SO, Rodland KD, Heffron F, Smith RD (2006) Analysis of the *Salmonella* Typhimurium proteome through environmental response toward infectious conditions. MolCel Proteomics 5:1450–1461

Agoston R, Soni K, Jesudhasan PR, Russell WK, Mohacsi-Farkas C, Pillai SD (2009) Differential expression of proteins in *Listeria monocytogenes* under thermotolerance-inducing, heat shock, and prolonged heat shock conditions. Foodborne Pathog Dis 6:1133–1140

Arroyo-López FN, Querol A, Bautista-Gallego J, Garrido-Fernández A (2008) Role of yeasts in table olive production. Int J Food Microbiol 128:189–196

Aydemir O, Harth H, Weckx S, Dervişoğlu M, De Vuyst L (2015) Microbial communities involved in Kaşar cheese ripening. Food Microbiol 46:587–595

Bardakci F (2001) Random amplified polymorphic DNA (RAPD) markers. Turk J Biol 25:2185–2196

Birren B, Lai E (2012) Pulsed field gel electrophoresis: a practical guide. Academic Press, San Diego, CA, pp. 1–6

Bisha B, Brehm-Stecher BF (2010) Combination of adhesive-tape-based sampling and fluorescence in situ hybridization for rapid detection of *Salmonella* on fresh produce. J Vis Exp 44: pii2308

Bonar E, Wojcik I, Wladyka B (2015) Proteomics in studies of *Staphylococcus aureus* virulence. Acta Bioch Pol 62:367–381

CDC (Center for Disease Control and Prevention) (2015) Shiga toxin producing *E. coli* & Food safety. Available via http://www.cdc.gov/features/ecoliinfection. Cited 28 Feb 2015

Centeno JA, Carballo J (2014) Starter and adjunct microbial cultures used in the manufacture of fermented and/or cured or ripened meat and dairy products. In: Ravishankar RV, Jamuna AB (eds) Beneficial microbes in fermented and functional foods. CRC Press, New York, pp 35–54

Ceuppens S, Li D, Uyttendale M, Renault P, Ross P, Ranst MV, Cocolin L, Donaghy J (2014) Molecular methods in food safety microbiology: interpretation and implications of nucleic acid detection. Compr Rev Food Sci F 13:551–577

Chen J, Tang J, Liu J, Cai Z, Bai X (2012) Development and evaluation of a multiplex PCR for simultaneous detection of five foodborne pathogens. J Appl Microbiol 112:823–830

Chen TY, Kuo SH, Chen ST, Hwang DF (2016) Differential proteomics to explore the inhibitory effects of acidic, slightly acidic electrolysed water and sodium hypochlorite solution on *Vibrio parahaemolyticus*. Food Chem 194:529–537

Cho Y, Park S, Barate AK, Truong QL, Han JH, Jung CH, Yoon JW, Cho S, Hahn TW (2015) Proteomic analysis of outer membrane proteins in *Salmonella enterica* Enteritidis. J Microbiol Biotechnol 25:288–295

Correia S, Nunes-Miranda JD, Pinto L, Santos HM, de Toro M, Sáenz Y, Torres C, Capelo JL, Poeta P, Igrejas G (2014) Complete proteome of a quinolone-resistant *Salmonella* Typhimurium phage type DT104B clinical strain. Int J MolSci 15:14191–14219

Dan T, Liu W, Sun Z, Lv Q, Xu H, Song Y, Zhang H (2014) A novel multi-locus sequence typing (MLST) protocol for *Leuconostoc lactis* isolates from traditional dairy products in China and Mongolia. BMC Microbiol 14:150. doi:10.1186/1471-2180-14-150

De Cesare A, Mioni R, Manfreda G (2007) Prevalence of *Listeria monocytogenes* in fresh and fermented Italian sausages and ribotyping of contaminating strains. Int J Food Microbiol 120:124–130

Domon B, Aebersold R (2010) Options and considerations when selecting a quantitative proteomics strategy. Nat Biotechnol 28:710–721

Donaldson JR, Nanduri B, Burgess SC, Lawrence ML (2009) Comparative proteomic analysis of *Listeria monocytogenes* strains F2365 and EGD. Appl Environ Microbiol 75:366–373

Donaldson JR, Nanduri B, Pittman JR, Givaruangsawat S, Burgess SC, Lawrence ML (2011) Proteomic expression profiles of virulent and avirulent strains of *Listeria monocytogenes* isolated from macrophages. J Proteomics 74:1906–1917

El-Hadedy D, El-Nour SA (2012) Identification of *Staphylococcus aureus* and *Escherichia coli* isolated from Egyptian food by conventional and molecular methods. J Genet Eng Biotechnol 10:129–135

Elmi A, Watson E, Sandu P, Gundogdu O, Mills DC, Inglis NF (2012) *Campylobacter jejuni* outer membrane vesicles play an important role in bacterial interactions with human intestinal epithelial cells. Infect Immun 80:89–98

Enany S, Yoshida Y, Yamamoto T (2014) Exploring extra cellular proteins in methicillin susceptible and methicillin resistant *Staphylococcus aureus* by liquid chromatography-tandem mass spectrometry. World J Microbiol Biotechnol 30:1269–1283

Encheva V, Gharbia SE, Wait R, Begum S, Shah HN (2005) Proteome analysis of serovars Typhimurium and Pullorum of *Salmonella enterica* subspecies I using two dimensional gel electrophoresis and LC/MS-MS. BMC Microbiol 5:42. doi:10.1186/1471-2180-5-42

Encheva V, Wait R, Begum S, Gharbia SE, Shah HN (2007) Protein expression diversity amongst serovars of Salmonella enterica. Microbiol 153:4183–4193

Ercolini D, Hill PJ, Dodd CE (2003) Development of a fluorescence in situ hybridization method for cheese using a 16S rRNA probe. J Microbiol Met 52:267–271

Espinosa JC, Fernandez-Gonzalez M, Ubeda J, Briones A (2002) Identification of wine yeasts by PCR-RFLP without previous isolation on plate. Food Technol Biotechnol 40:157–160

Fang Q, Brockmann S, Botzenhart K, Wiedenmann A (2003) Improved detection of *Salmonella* spp. in foods by fluorescent in situ hybridization with 23S rRNA probes: a comparison with conventional culture methods. J Food Protect 66:723–731

Favier GI, Estrada CSL, Otero VL, Escudero ME (2013) Prevalence, antimicrobial susceptibility, and molecular characterization by PCR and pulsed field gel electrophoresis (PFGE) of *Salmonella* spp. isolated from foods of animal origin in San Luis, Argentina. Food Control 29:49–54

Feng Y, Liu SL (2012) Pathogenic *Salmonella*. In: Yan X, Juneja VK, Fratamico PM, Smirth JL (eds) Omics, microbial modelling and technologies for foodborne pathogens. DEStech Publications, Lancaster, PA, pp 43–68

Foley SL, Grant K (2007) Molecular techniques of detection and discrimination of foodborne pathogens and their toxins. In: Simjee S (ed) Foodborne diseases. Humana Press, Totowa, NJ, pp 485–510

França LT, Carrilho E, Kist TB (2002) A review of DNA sequencing techniques. Q Rev. Biophysics 35:169–200

Fratamico PM, Bayles DO (2005) Molecular approaches for detection, identification, and analysis of food borne pathogens. In: Fratamico PM, Bhunia AK, Smith JL (eds) Foodborne pathogens, microbiology and molecular biology. Caister Academic Press, Norfolk, pp 1–3

Fuchizawa I, Shimizu S, Kawai Y, Yamazaki K (2008) Specific detection and quantitative enumeration of *Listeria* spp. using fluorescent in situ hybridization in combination with filter cultivation (FISHFC). J Appl Microbiol 105:502–509

Fusco V, Quero GM (2012) Nucleic acid-based methods to identify, detect and type pathogenic bacteria occurring in milk and milk products. In: Eissa AA (ed) Structure and function of food engineering. In Tech, Rijeka. doi:10.5772/49937

Giraffa G, Carminati D (2008) Molecular techniques in food fermentation: principles and applications. In: Cocolin L, Ercolini D (eds) Molecular techniques in the microbial ecology of fermented foods. Springer Science & Business Media, New York, pp 1–30

Haryani Y, Noorzaleha AS, Fatimah AB, Noorjahan BA, Patrick GB, Shamsinar AT, Son R (2007) Incidence of *Klebsiella pneumonia* in street foods sold in Malaysia and their characterization by antibiotic resistance, plasmid profiling, and RAPD–PCR analysis. Food Control 18:847–853

Hayashi K (2012) Manupilation of DNA by PCR. In: Mullis KB, Ferre F, Gibbs RA (eds) The polymerase chain reaction. Springer Science & Business Media, New York, pp 1–4

He L, Deng QL, Chen MT, Wu QP, Lu YJ (2015) Proteomics analysis of *Listeria monocytogenes* ATCC 19115 in response to simultaneous triple stresses. Arch Microbiol 197:833–841

Huang DH, Wang K, Chiu CP, Pan TM, Tsai TY (2015) Effects of chemical and low-temperature treatments and adaption on the responses of virulence factor genes and outer membrane proteins in *Escherichia coli* O157:H7. J Microbiol Immunol Infect 48:604–612

Huang G, Mason SL, Hudson JA, Clerens S, Plowman JE, Hussain MA (2014) Proteomic differences between *Listeria monocytogenes* isolates from food and clinical environments. Pathogens 3:920–933

Huang Y-J, Tsai T-Y, Pan T-M (2007) Physiological response and protein expression under acid stress of *Escherichia coli* O157:H7 TWC01 isolated from Taiwan. J Agric Food Chem 55:7182–7191

Hu WS, Lin YH, Shih CC (2007) A proteomic approach to study *Salmonella enterica* serovar Typhimurium putative transporter YjeH associated with ceftriaxone resistance. Biochem Biophys Res Commun 361:694–699

Jang K-S, Sweredoski MJ, Graham RLJ, Hess S, Clemons WM Jr (2014) Comprehensive proteomic profiling of outer membrane vesicles from *Campylobacter jejuni*. J Proteomics 98:90–98

Karatzas KA, Randall LP, Webber M, Piddock LJ, Humphrey LJ, Woodard MJ, Coldham NG (2008) Phenotypic and proteomic characterization of multiply antibiotic-resistant variants of

Salmonella enterica serovar Typhimurium selected following exposure to disinfectants. Appl Environ Microbiol 74:1508–1516

Kaur P, Chakraborti A (2010) Proteome analysis of a food borne pathogen enteroaggregative *Escherichia coli* under acid stress. J Proteomics Bioinform 3:010–019

Keeratipibul S, Techaruwichit P (2012) Tracking sources of *Listeria* contamination in a cooked chicken meat factory by PCR-RAPD-based DNA fingerprinting. Food Control 27:64–72

Killer J, Skřivanová E, Hochel I, Marounek M (2015) Multilocus sequence typing of *Cronobacter* strains isolated from retail foods and environmental samples. Foodborne Pathog Dis 12:514–521

Klose J (1975) Protein mapping by combined isoelectric focusing and electrophoresis in mouse tissues. Humangenetik 26:231–243

Kumar NS, Gurusubramanian G (2011) Random amplified polymorphic DNA (RAPD) markers and its applications. Sci Vis 11:116–124

Kurtzman CP (2015) Identification of food and beverage spoilage yeasts from DNA sequence analyses. Int J Food Microbiol 213:71–78

Kusch H, Engelmann S (2014) Secrets of the secretome in *Staphylococcus aureus*. Int J Med Microbiol 304:133–141

Lee E-Y, Choi D-Y, Kim D-K, Kim J-W, Park JO, Kim S, Kim S-H, Desiderio DM, Kim Y-K, Kim K-P, Gho YS (2009) Gram-positive bacteria produce membrane vesicles: Proteomics-based characterization of *Staphylococcus aureus*-derived membrane vesicles. Proteomics 9:5425–5436

Levin RE (2009) Rapid detection and characterization of foodborne pathogens by molecular techniques. CRC Press, New York, pp. 1–161

Lippolis JD, Bayles DO, Reinhardt TA (2009) Proteomic changes in *Escherichia coli* when grown in fresh milk versus laboratory media. J Proteome Res 8:149–158

Li W, Raoult D, Fournier P-E (2009) Bacterial strain typing in the genomic era. FEMS Microbiol Rev 33:892–916

Lucena-Padrós H, Caballero-Guerrero B, Maldonado-Barragán A, Ruiz-Barba JL (2014) Microbial diversity and dynamics of Spanish-style green table-olive fermentations in large manufacturing companies through culture-dependent techniques. Food Microbiol 42:154–165

Mackay IM, Mackay JF, Nissen MD, Sloots TP (2007) Real-time PCR: history and fluorogenic chemistries In: Mackay IM (ed) Real-time PCR in microbiology: from diagnosis to characterization. Caister Academic Press, Norfolk, pp. 1–40

Maiden MCJ, Bygraves JA, Feil E (1998) Multilocus sequence typing: a portable approach to the identification of clones within populations of pathogenic microorganisms. Proc Natl Acad Sci USA 95:3140–3145

Marcotte EM (2007) How do shotgun proteomics algorithms identify proteins? Nat Biotechnol 25:755–757

Marty E, Buchs J, Eugster-Meier E, Lacroix C, Meile L (2012) Identification of staphylococci and dominant lactic acid bacteria in spontaneously fermented Swiss meat products using PCR–RFLP. Food Microbiol 29:157–166

Miyamoto KN, Monteiro KM, Caumo KS, Lorenzatto KR, Ferreira HB, Brandelli A (2015) Comparative proteomic analysis of *Listeria monocytogenes* ATCC 7644 exposed to a sublethal concentration of nisin. J Proteomics 119:230–237

Monteiro R, Vitorino R, Domingues P, Radhouani H, Carvalho C, Poeta P, Torres C, Igrejas G (2012) Proteome of a methicillin-resistant *Staphylococcus aureus* clinical strain of sequence type ST398. J Proteomics 75:2892–2915

Muñoz R, Rivas B, Curiel JA (2014) Multilocus sequence typing of food microorganisms. In: Batt CA (ed) Encyclopedia of food microbiology. Academic Press, London, pp 300–310

Muñoz-Atienza E, Araújo C, Del Campo R, Hernández PE, Herranz C, Cintas LM (2016) Safety assessment and molecular genetic profiling by pulsed-field gel electrophoresis (PFGE) and PCR-based techniques of *Enterococcus faecium* strains of food origin. LWT-Food Sci Technol 65:357–362

O'Farrell PH (1975) High resolution two-dimensional electrophoresis of proteins. J Biol Chem 250:4007–4021

Olive DM, Bean P (1999) Principles and applications of methods for DNA-based typing of microbial organisms. J Clin Microbiol 37:1661–1669

Pereira EL, Ramalhosa E, Borges A, Pereira JA, Baptista P (2015) YEAST dynamics during the natural fermentation process of table olives (*Negrinha de Freixo*cv.). Food Microbiol 46:582–586

Pernthaler J, Glöckner F-O, Schönhuber W, Amann R (2001) Fluorescence in situ hybridization (FISH) with rRNA-targeted oligonucleotide probes. Method Microbiol 30:207–210

Pham T, Wimalasena T, Box WG, Koivuranta K, Storgårds E, Smart KA, Gibson BR (2011) Evaluation of ITS PCR and RFLP for differentiation and identification of brewing yeast and brewery wild'yeast contaminants. J Inst Brew 117:556–568

Pinto L, Poeta P, Vieira S, Caleja C, Radhouani H, Carvalho C, Vieira-Pinto M, Themudo P, Torres C, Vitorino R, Domingues P, Igrejas G (2010) Genomic and proteomic evaluation of antibiotic resistance in *Salmonella* strains. J Proteomics 73:1535–1541

Plengvidhya V, Breidt F, Fleming HP (2004) Use of RAPD-PCR as a method to follow the progress of starter cultures in sauerkraut fermentation. Int J Food Microbiol 93:287–296

Rapley R, Whitehouse D (2007) Basic tools and techniques in molecular biology. In: Rapley R, Whitehouse D (eds) Molecular forensics. Wiley, West Sussex, pp 21–34

Resch A, Leicht S, Saric M, Pasztor L, Jakob A, Gotz F, Nordheim A (2006) Comparative proteome analysis of *Staphylococcus aureus* biofilm and planktonic cells and correlation with transcriptome profiling. Proteomics 6:1867–1877

Rivoal K, Fablet A, Courtillon C, Bougeard S, Chemaly M, Protais J (2013) Detection of *Listeria* spp. in liquid egg products and in the egg breaking plants environment and tracking of *Listeria monocytogenes* by PFGE. Int J Food Microbiol 166:109–116

Rodríguez A, Luque MI, Andrade MJ, Rodríguez M, Asensio MA, Córdoba JJ (2011) Development of real-time PCR methods to quantify patulin-producing molds in food products. Food Microbiol 28:1190–1199

Rodriguez-Lazaro D, Gonzalez-García P, Delibato E, De Medici D, García-Gimeno RM, Valero A, Hernandez M (2014) Next day *Salmonella* spp. detection method based on real-time PCR for meat, dairy and vegetable food products. Int J Food Microbiol 184:113–120

Salustiano VC, Andrade NJ, Soares NFF, Lima JC, Bernardes PC, Luiz LMP, Fernandes PE (2009) Contamination of milk with *Bacillus cereus* by post-pasteurization surface exposure as evaluated by automated ribotyping. Food Control 20:439–442

Saron T (2007) Legal aspects of food safety. In: Wirtanen G, Salo S (eds) Microbial contaminants & contamination routes in food industry. Finland, VTT, pp 51–55

Scallan E, Hoekstra RM, Angulo FJ, Tauxe RV, Widdowson M-A, Roy SL, Griffin PM (2011) Foodborne illness acquired in the United States—major pathogens. Emerg Infect Dis 17:7–15

Scharff RL (2012) Economic burden of health losses due to foodborne illness in the United States. J Food Protect 1:123–131

Scott NE, Cordwell SJ (2009) *Campylobacter* proteomics: guidelines, challenges and future perspectives. Expert Rev Proteomics 6:61–74

Seseña S, Sánchez I, Palop L (2005) Characterization of *Lactobacillus* strains and monitoring by RAPD-PCR in controlled fermentations of "Almagro" eggplants. Int J Food Microbiol 104:325–335

Shin S, Lu G, Cai M, Kim K-S (2005) *Escherichia coli* outer membrane protein A adheres to human brain microvascular endothelial cells. Biochem Biophys Res Commun 330:1199–1204

Singh P, Mustapha A (2015) Multiplex real-time PCR assays for detection of eight Shiga toxin-producing *Escherichia coli* in food samples by melting curve analysis. Int J Food Microbiol 215:101–108

Soka S, Susanta A (2010) Genetic diversity of yeasts from fermented orange juice based on PCR-RFLP and sequence analysis of the internal transcribed spacer regions. Microbiol Indonesia 4:1–5

Stender H, Oliveira K, Rigby S, Bargoot F, Coull J (2001) Rapid detection, identification, and enumeration of Escherichia coli by fluorescence in situ hybridization using an array scanner. J Microbiol Method 45:31–39

Stjepanovic A, Østlie HM, Skeie SB (2011) Novel food pathogen testing technologies: molecular biology methods. In: Today science-tomorrow industry. International scientific and professional conference. 13th Ružička Days, Vukovar, Sept 16–17, pp 55–68

Sun J-S, Hahn T-W (2012) Comparative proteomic analysis of *Salmonella enterica* serovars Enteriditis, Typhimurium, and Gallinarum. J Vet Med Sci 74:281–295

Ye Y, Gao J, Jiao R, Li H, Wu Q, Zhang J, Zhong X (2015) The membrane proteins involved in virulence of *Cronobacter sakazakii* virulent G362 and attenuated L3101 isolates. Front Microbiol 6:1238. doi:10.3389/fmicb.2015.01238

Yu J, Sun Z, Liu W, Xi X, Song Y, Xu H, Lv Q, Bao Q, Menghe B, Sun T (2015) Multilocus sequence typing of *Streptococcus thermophilus* from naturally fermented dairy foods in China and Mongolia. BMC Microbiol 15:236. doi:10.1186/s12866-015-0551-0

Yu Q, Zhai L, Bie X, Lu Z, Zhang C, Tao T, Zhao H (2016) Survey of five food-borne pathogens in commercial cold food dishes and their detection by multiplex PCR. Food Control 59:862–869

Zahavi T, Droby S, Cohen L, Weiss B, Ben-Ari R (2015) Characterization of the yeast flora on the surface of grape berries in Israel. Vitis 41:203–208

Zhou Z, Li X, Liu B, Beutin L, Xu J, Ren Y, Feng L, Lan R, Reeves PR, Wang L (2010) Derivation of *Escherichia coli* O157:H7 from its O55:H7 precursor. PLoS One 5:8700. doi:10.1371/journal.pone.0008700

Zhu W, Smith JW, Huang C-M (2010) Mass spectrometry based label-free quantitative proteomics. J Biomed Biotechnol 2010:840518. doi:10.1155/2010/840518

Chapter 3
Strategies of Mass Cultivation of Microbes: *Sebacinales*

Diksha Bhola and Varma Ajit

Abstract The order *Sebacinales* placed at the base of *Basidiomycetes* exhibits fairly large diversity of mutualistic fungi. The rDNA sequence-based molecular ecology studies divulge that the members of *Sebacinales* form mutualistic associations with a number of terrestrial plant species. One of the extensively known members of *Sebacinales* is *Piriformospora indica*, which is an AM-like mycorrhizal fungus. It exhibits growth-promoting characteristics and can be cultured axenically. Various complex media are employed for the growth of the fungus; however, a simple and cheap medium was developed for commercial production. The optimisation of the media based on nutrient requirements had elucidated that Hill and Kaefer medium is the optimal medium for the growth of fungus. Fungal cultures can be raised on large scale in rotary shakers as well as fermenters. Further the fungal cultures are also produced on large scale as nanomycorrhiza.

3.1 Introduction

More than 80 % of known terrestrial plant species form symbiotic associations with mutualistic mycorrhizal fungi (Brundrett 2009). A wide diversity of these fungi belongs to the order *Sebacinales*. Though exhibiting a fairly large diversity of mutualistic fungi in comparison to other orders, *Sebacinales* is rather a recently described order (Weiß et al. 2004; Glen et al. 2002; Urban et al. 2003; Selosse et al. 2002, 2007; Kottke et al. 2003; Setaro et al. 2006; McKendrick et al. 2002). The order *Sebacinales* is placed at the base of *Basidiomycetes* and exhibits evident phylogenetic affinity *Geastrum* spp. It has been postulated that ECM is the proposed ancestor of both *Geastrum* and *Sebacinales* clade or the entire *Hymenomycetes* (Taylor et al. 2003; Weiß et al. 2004; Smith and Read 1997).

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The rDNA sequence-based molecular ecology studies divulge that members of *Sebacinales* form mutualistic associations with a number of terrestrial plant species. The sequence analysis of members of *Sebacinales* obtained from different habitats reveals that the order segregates into two clades, A and B, that vary in their ecology (Weiß et al. 2004). Fruit bodies are unique to clade A of order *Sebacinales*. Thus clade A sequence is acquired from fruit bodies, orchids as well as many trees, while clade B of order *Sebacinales* is obtained from autotrophic orchids, liverworts and Ericaceae (Varma et al. 2013). The molecular phylogenetic evaluation states that the order belongs to *Basidiomycetes*, yet a very few basidiome-producing species have been described (Hibbett et al. 2007). On the basis of the molecular phylogenetic analysis, the *Sebacinales* are isolated from the rest of the *Auriculariales sensu* Bandoni taxa despite their macroscopial as well as ultrastructural similarities (Varma et al. 2013).

Similarly the molecular phylogenetic studies revealed that *P. indica* is a member of the order *Sebacinales*. The partial 18S rDNA sequence analysis placed *P. indica* in *Basidiomycota* close to the *Rhizoctonia solani* group (Varma et al. 2013). A maximum likelihood analysis of 18S rDNA sequence confirmed these postulations. Further according to their similarities to *Zygomycetes*, *P. indica* is termed as an AM-like fungus (Franken et al. 2000). Another fungus, *Sebacina vermifera* sensu, containing 20S rRNA is placed in the *Sebacinaceae* family.

Thus *Sebacinales* is an extensively versatile order consisting of symptomless endophytes (Weiß et al. 2011). The members of *Sebacinales* have positive effects on the growth, resistance against biotic and abiotic stress as well as yield of the plants they are associated with (Deshmukh et al. 2006; Waller et al. 2005). Thus it enables these fungal spp. to be a feasible source of biofertilisers and biocontrol agents.

3.2 Morphology and Taxonomy of *Sebacinales*

The fungal species belonging to *Sebacinales* such as *P. indica* have white to almost hyaline hyphae. The hyphae are thin walled and have a diametric range of 0.7–3.5 μm. The hyphae are irregularly septate and often exhibit anastomosis. The highly interwoven hyphae appear as intermingled cords and branch irregularly. External deposits, polysaccharides or hydrophobic proteins can be noticed on hyphal walls at regular intervals. The irregular septation of hyphae accounts for the presence of more than one nuclei in a single compartment. The distinct chlamydospores appear singly or in clusters. Initially the chlamydospores are thin walled and hyaline, while they become thick walled towards maturity. Further no sexual structures or clamp connections were observed (Varma et al. 2001). The mycelium has a subsurfaced and concentric growth on agar medium. When grown on solid culture media, very few aerial hyphae were formed. Occasionally the mycelium fabricates periodic rings on agar medium, whereas the structure of the mycelium was homogenous. The morphological characteristics of the mycelium

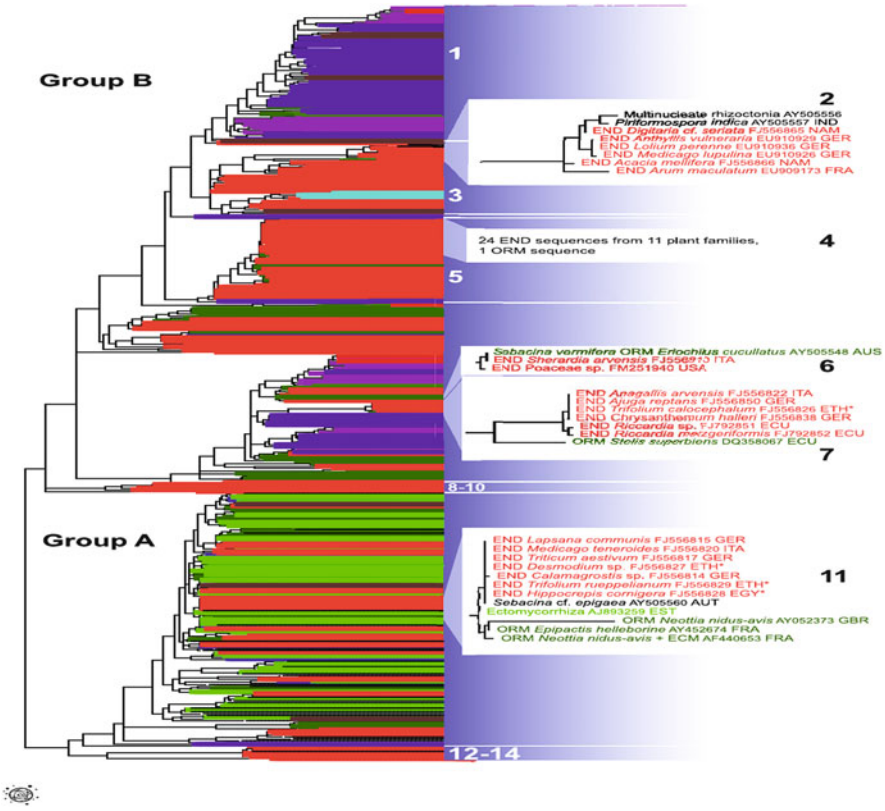

Fig. 3.1 Phylogenetic relationships of *Sebacinales* based on maximum likelihood analysis of partial nuclear-encoded ribosomal large subunit sequences cited from Weiß et al. (2011)

greatly differ with variations in conditions of cultivation or nutrient compositions of the culture medium.

Through 18 s RNA molecular methods and electron microscopy, it was elucidated that this fungus belongs to *Hymenomycetes*. With the help of electron microscopy, the presence of dolipores with non-perforated parenthesomes was elucidated. This further implied that *P. indica* belongs to *Hymenomycetes* (*Basidiomycota*). Sequence comparison showed the close relation of *P. indica* with *Rhizoctonia* group (Varma et al. 2001). *P. indica* is placed as a member of the *Basidiomycetes* order *Sebacinales* by the molecular phylogenetic analysis (Hibbett et al. 2007; Qiang et al. 2012; Weiß et al. 2004). During the molecular ecology studies, the sebacinoid fungi are frequently detected as mycobionts of plant roots, thus pronouncing that *Sebacinales* retain massive biodiversity (Weiß et al. 2011). Even though the exact phylogenetic position of the *Sebacinales* is still not very evident in *Agaricomycetes*, it is divisible into two major clades which have been designated as A and B (Fig. 3.1) (Qiang et al. 2012; Weiß et al. 2011). The anamorphic *P. indica* is associated with group B. Basiewicz et al. inferred

significant changes in physiological and molecular parameters within similar strains of *Piriformospora* (Basiewicz et al. 2012). Further *Piriformospora williamsii* was described as a new member of the genus *Piriformospora*.

3.3 Morphogenesis of *P. indica*

The morphogenesis of the fungus was monitored for about 7 days in batch cultures. The cylindrical hyphae after 2 days started to enlarge at places, and after 5 days many of the hyphae turned into aggregated spores. At the end of 7 days, typical pear-shaped spores were produced in abundance. In another experiment, after 4 days of incubation at 28 °C the fungal culture was transferred to cold conditions at 4 °C and massive sporulation was found within 24 hours. Vice versa the cultures after 25 s were incubated at 37 °C, and the massive sporulation was recorded (Fig. 3.2).

3.4 Nutrient Requirements for Growth

Carbon is very essential for fungal metabolism as the fungi are heterotrophic for carbon compounds. The carbon taken up by the fungus provides two crucial functions. First carbon is required for the synthesis of cell components. Secondly, energy is obtained by the oxidation of compounds of carbon. The fungi can utilise a variety of carbon sources such as monosaccharide, disaccharides, oligosaccharides, polysaccharides, organic acids and lipids. On supplementation of fungal cultures with glucose, fructose and sucrose, glucose is consumed first, whereas the sucrose is metabolised by invertase. Thus first all free glucose is consumed, and after this slow utilisation of fructose takes place.

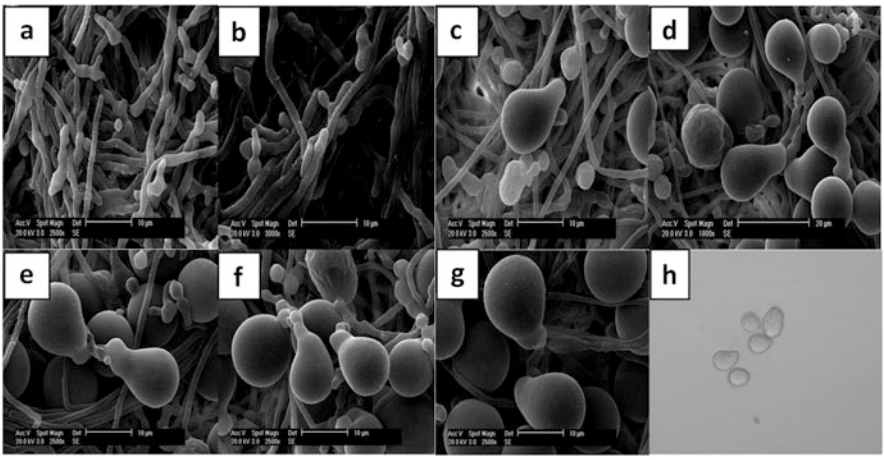

Fig. 3.2 Morphogenesis of *P. indica*

Individual amino acids also have distinctive effects on fungal growth. On addition of glycine, methionine, serine and alanine, promotion of growth of fungus to different extents can be observed. However, not a significant change is observed in fungal growth when augmented with glutamine, asparagine and histidine. Phosphorous is paramount for the growth of members of *Sebacinales*. The fungus utilises a wide range of inorganic and organic phosphate sources. Acid phosphatases were observed to be active in *P. indica* mycelium (Varma et al. 2001).

3.5 Media Composition

A large variety of synthetic and complex media are employed for the activation of fungal strains.

(a) MMN 1/10 (Herrmann et al. 1998)

Composition	g/l
$CaCl_2 \cdot 2H_2O$	0.07
$MgSO_4 \cdot 7H_2O$	0.15
NaCl	0.03
$(NH_4)_2HPO_4$	0.03
KH_2PO_4	0.05

Trace elements	mg/l
$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	0.09
H_3BO_4	1.55
$CuSO_4 \cdot 5H_2O$	0.13
KCl	3.73
$MnSO_4 \cdot H_2O$	0.84
$ZnSO_4 \cdot 7H_2O$	0.58

Fe-EDTA	mg/l
FeSO4	8.50
EDTA	1.50
Agar	20.0 g

(b) Modified Aspergillus Medium (Varma et al. 2001)

The media composition was the same, except that yeast extract, peptone and casamino acid were reduced to 1/10 in quantity

(c) M4 N (Mukerji et al. 1998)

Composition	g/l
D-Glucose	10.0
$(NH_4)2HPO_4$	0.25

(continued)

Composition	g/l
KH_2PO_4	0.50
$MgSO_4 \cdot 7H_2O$	0.15
$CaCl_2 \cdot 2H_2O$	0.05
Ferric citrate (2 % ferric citrate, 2 % citric acid v/v)	7.0 ml
NaCl	0.025
Thiamine HCl	100.0 mg
MES	2.5
Malt extract	1.5
Yeast extract	1.5
Agar	15.0
pH	5.6

(d) MMNC (Kottke et al. 1987; Marx 1969)

Composition	g/l
Glucose	10.0
$CaCl_2 \cdot 2H_2O$	0.07
$MgSO_4 \cdot 7H_2O$	0.15
NaCl	0.03
$(NH_4)2HPO_4$	0.25
KH_2PO_4	0.5
Casein-hydrolysate	1.0
Malt extract	5.0

Trace elements	mg/l
$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	0.02
H_3BO_4	1.55
$CuSO_4 \cdot 5H_2O$	0.13
KCl	3.73
$MnSO_4 \cdot H_2O$	0.85
$ZnSO_4 \cdot 7H_2O$	0.58

Fe-EDTA	mg/l
$FeSO_4$	8.5
EDTA	1.5

Vitamins	mg/l
Thiamine	0.1
Riboflavin	0.1
pH	5.6
Agar	20.0 g

(e) MS (Murashige and Skoog 1962)

Chemicals	mg/l
Macronutrients	
NH_4NO	30.5
KNO_3	1650.0
$CaCl_2 \cdot 2H_2O$	900.0
$MgSO_4 \cdot 7H_2O$	440.0
KH_2PO_4	370.0

Micronutrients	
KI	170.0
H_3BO_3	0.83
$MnSO_4 \cdot H_2O$	6.20
$ZnSO_4 \cdot 7H_2O$	15.60
$NaMoO_4 \cdot 2H_2O$	8.60
$CuSO_4 \cdot 5H_2O$	0.25
$CoCl_2 \cdot H_2O$	0.025

Iron source	
$Na_2 \cdot EDTA$	0.025
$FeSO_4 \cdot 7H_2O$	37.30

Vitamins	
Nicotinic acid	27.8
Pyridoxine HCl	0.5
Thiamine HCl	0.1
Glycine	2.0
Myo-inositol	100.0
Agar	0.7 % (w/v)
Sucrose	3.0 % (w/v)
PH	5.6–5.7

Each chemical was dissolved in bidistilled water individually. pH of the medium was adjusted using 1 N NaOH/HCl before autoclaving at 121 °C, 15 lbs. for 20 min. Stock solutions were stored at 4 °C except organic supplements, which were stored at 20 °C.

(f) WPM ('Woody Plant Medium' for Populus) Ahuja (1986)

Composition	g/l
Sucrose	20.0
K_2SO_4	1.00
$Ca\ (NO_3)_2 \cdot 4H_2O$	0.73
NH_4NO_3	0.40
$MgSO_4 \cdot 7H_2O$	0.37
Myo-inositol	0.10
Agar	7.00

Add 700 ml H_2O, and adjust pH to 5.8 using 3.7 % HCl (ca. 9.5 ml).
Add after autoclaving sterile phosphate solution (0.17 g KH_2PO_4 dissolved in 270 ml H_2O) + 15 ml NaOH (saturated)
10 ml of trace element stock solution (see below)
10 ml Fe-EDTA (see below)
10 ml glycine stock solution (100×: solve 20 mg in 100 ml)
1 ml thiamine stock solution (1000×: solve 10 mg in 100 ml)
1 ml nicotinic acid stock solution (1000×: solve 50 mg in 100 ml)
1 ml $CaCl_2$ stock solution (1000×: solve 3.6 g in 50 ml)
250 ml pyridoxine stock solution (4000×: solve 40 mg in 100 ml)
100 ml $CuSO_4$ stock solution (10,000×: solve 25 mg in 100 ml)
Sterilise by filtration before adding

100× trace element stock solution (g/l, autoclave, store at 4 °C):

$MnSO_4 \cdot H_2O$	2.23
$ZnSO_4 \cdot 7H_2O$	0.86
H_3BO_4	0.62
Ammonium molybdate	0.10
KI	0.09

100× Fe-EDTA stock solution.

Dissolve 0.128 g $FeSO_4$ and 0.172 g EDTA at 60 °C in 100 ml H_2O and store at 4 °C, 20 g.

$CaCl_2 \cdot 2H_2O$	0.07
$MgSO_4 \cdot 7H_2O$	0.15
NaCl	0.03
$(NH_4)_2HPO_4$	0.03
KH_2PO_4	0.05

Trace elements	mg/l
$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	0.018
H_3BO_4	

(g) MMN (Modified Melin-Norkrans) (Johnson et al. 1957)

Composition	g/l
NaCl	0.025
KH_2PO_4	0.5
$(NH_4)_2HPO_4$	0.25
$CaCl_2$	0.05
$MgSO_4$	0.15
$FeCl_3$	0.001
Thiamine hydrochloride	83.0 ml
Trypticase peptone	0.1 % (w/v)
Glucose monohydrate	1.0 % (w/v)

(continued)

Composition	g/l
Malt extract	5.0 % (w/v)
Trace elements from stock	10.0 ml/l

Trace elements (stock)	g/l
KCl	3.73
H_3BO_3	1.55
$MnSO_4 \cdot H_2O$	0.85
$ZnSO_4$	0.56
$CuSO_4$	0.13

pH was adjusted to 5.8 with 1 N HCl/NaOH.

All the stocks were stored at 4 °C except thiamine hydrochloride which was stored at 20 °C.

(h) Malt Extract (Gallowey and Burgess 1962)

Composition	g/l
Malt extract	30.0
Mycological peptone	5.0
Agar	15.0
pH	5.4

(i) Potato Dextrose Agar (PDA) (Martin 1950)

Composition	g/l
Potato peel	200.0
Dextrose	20.0
Agar	15.0
Distilled water	1.0

Skin of potatoes was peeled off, cut into small pieces and boiled (200 g) in 500 ml of water, till they were easily penetrated by a glass rod. The boiled water containing potato peel was filtered through cheesecloth and dextrose was added to the filtrate. Agar was dissolved and the required volume (1 l) was made up by the addition of water. The medium was autoclaved at 15-lb. pressure for 20 ml.

(j) Aspergillus Medium (Kaefer 1977)

Composition	g/l
Glucose	20.0
Peptone	02.0
Yeast extract	01.0
Casamino acid	01.0
Vitamin stock solution	01.0 ml
Macroelements from stock	50.0 ml
Microelements from stock	02.5 ml
Agar	10.0
$CaCl_2$ 0.1 M	1.0 ml

(continued)

Composition	g/l
$FeCl_3$ 0.1 M	1.0 ml
pH	6.5

Macroelements (major elements)	Stock (g/l)
$NaNO_3$	120.0
KCl	10.4
$MgSO_4 \cdot 7H_2O$	10.4
KH_2PO_4	30.4

Microelements (trace elements)	Stock (g/l)
$ZnSO_4 \cdot 7H_2O$	22.0
H_3BO_3	11.0
$MnCl_2 \cdot 4H_2O$	5.0
$FeSO_4 \cdot 7H_2O$	5.0
$CoCl_2 \cdot 6H_2O$	1.6
$CuSO_4 \cdot 5H_2O$	1.6
$(NH_4)_6Mo_7O_{27} \cdot 4H_2O$	1.1
Na_2EDTA	50.0

Vitamins	Percent
Biotin	0.05
Nicotinamide	0.5
Pyridoxal phosphate	0.1
Amino benzoic acid	0.1
Riboflavin	0.25

pH was adjusted to 6.5 with 1 N HCl. All the stocks were stored at 4 °C except vitamin which was stored at 20 °C.

(k) Jaggery

Composition	w/v
Grounded jiggery	4 %
pH	6.5

The grounded jiggery was dissolved in distilled water.

Chemical composition (in %) of jaggery is (Figs. 3.3 and 3.4):

Sucrose	60–85
Glucose and fructose	5–15
Protein	0.4
Fat	0.05
Minerals	0.6–1.0
Calcium	0.4
Magnesium and phosphorus	0.045
Iron	11

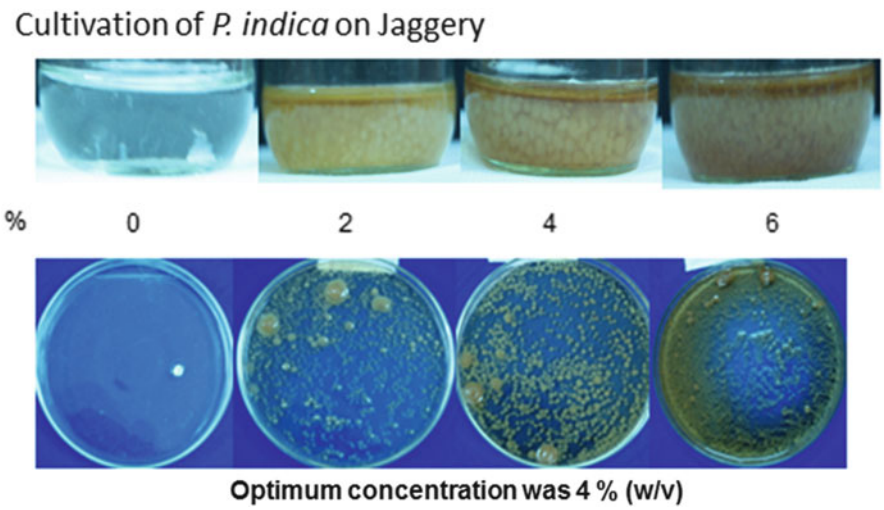

Fig. 3.3 Cultivation of *P. indica* on different concentrations of jaggery

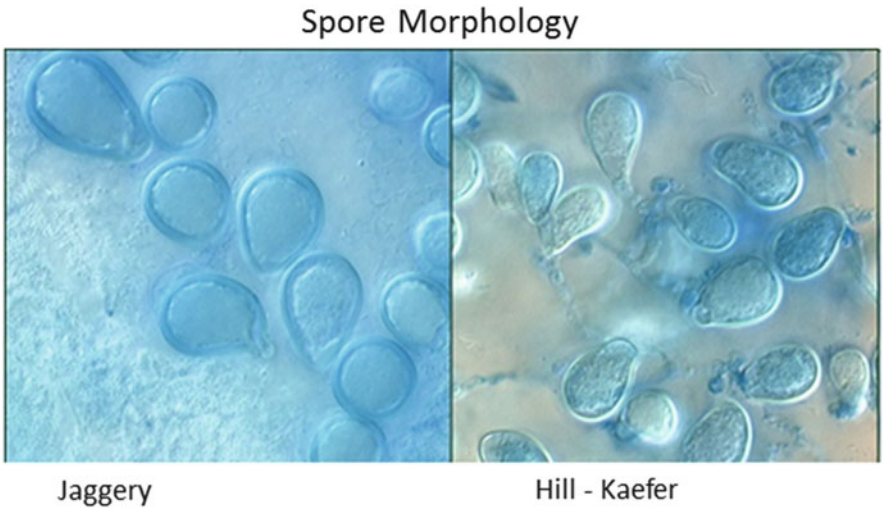

Fig. 3.4 Spore morphology of *P. indica* on jaggery and Hill-Kaefer medium

3.6 Growth in Batch Culture on Shaker

Many fungi effectively sporulate in submerged cultures containing suitable medium with optimised nutrients (Vezina et al. 1965). Thus fungal cultures can be produced aseptically on large scale using shake flasks. The sporulation can be enhanced by transferring the jar in cold for 24 h.

3.6.1 Preparation of Media

Various media compositions are employed for the production of fungal cultures in shake flaks. Hill and Kaefer medium along with other media such as YPG media, potato dextrose broth (PDB), and malt extract are effectively employed for the submerged cultivation of fungal cultures. The media are prepared following the standardised media compositions in distilled water. The desired pH of the composed media is adjusted using acid or base and quantified using a pH metre.

3.6.2 Sterilisation of Medium

Prior to inoculation of media for the production of cultures, the media should be sterilised. This avoids growth of undesired microbes along with the cultures. Sterilisation of medium is done under high-pressure saturated steam at 121 °C for 15–20 min in autoclaves.

3.6.3 Inoculum Preparation

The stock cultures of fungus are maintained on slants containing complex medium (Hill and Kaefer 2001) augmented with 15 g/L agar. The slants after being inoculated were incubated at 30 °C for 10 days and later on stored at 4 °C. For the preparation of inoculum, the fungal cultures were initially grown in a petri dish containing Kaefer medium (Kaefer 1977). At the time of inoculation, agar discs of approximately 8 mm were then punched out using sterilised corkborer. These discs were then used for inoculation of seed cultures.

3.6.4 Growth in Flasks on Shaker

The submerged cultures were raised in 500 mL of flasks containing 100 mL of complex media most probably Hill and Kaefer medium. The flasks were inoculated with 5 mL of freshly prepared inoculum at 30 °C under constant shaking at 200 rpm on a rotary shaker.

3.6.5 Harvesting of Biomass

After maximum growth is attained in the flasks, the biomass is harvested. The culture is filtered and the biomass is separated. The culture can also be centrifuged at 3000 rpm for separation of filtrate and biomass. The filtrate thus obtained can be employed for the study of bioactives produced by the fungal cultures. The biomass separated is used as a bioinoculant by mixing the 2 % biomass with sterilised magnesium sulphite or vermiculite.

3.7 Production of Fungal Cultures in Fermenters

Fermenters provide optimised environmental and nutritional conditions for the large-scale production of microbial cultures. The constant administration of conditions at variable stages in fermenters enables a more efficient scale-up of microbial cultures. The submerged conditions enhance the uptake of nutrients resulting in stimulation of the biochemical processes. Fermentation of the microbes can be accomplished through the following three processes, batch, continuous and fed-batch.

Batch culture comprises of a closed system which encompasses an initial restricted availability of nutrient. The batch fermentation is employed for the production of biomass as well as primary and secondary metabolites. Further in fed-batch systems, the exponential growth phase can be prolonged by the continuous addition of fresh culture to the system. This addition results in the continuous culture system. The continuous culture systems require media which is designed for substrate limited growth. Thus these systems effectively maintain microbial population in exponential growth where cultures grow at a constant rate and biomass concentration for extended periods (Lansing et al. 2005).

There is yet another system called the fed-batch system. The batch cultures constantly being fed with medium without the culture fluid being removed corresponds to the fed-batch culture (Yoshida et al. 1973). It is initially established in batch mode and further fed accordingly depending upon the conditions required by the culture. The fed-batch cultures control the organism's growth rate which is

related to the specific rate of oxygen uptake. Thus fed-batch culture systems are readily used in fermentation technology.

3.7.1 Medium for Optimal Growth

The media used for fermentation greatly influence the nutritional requirements as well as physiochemical environment and thus directly affect productivity and process economics (Zhang and Greasham 1999). Therefore a suitable media should invariably support vegetative growth and production of spores. The optimum growth conditions are observed in a modified Kaefer media with peptone, 3.0; yeast extract, 3.0; KH_2PO_4, 1.83; and $MgSO_4.7H_2O$, 0.65 g/L. The concentrations of other components were the same as in the original Kaefer medium without $NaNO_3$ and KCl, while the glucose concentration was 20 g/L (Kumar et al. 2011).

3.7.2 Sterilisation of the Fermenter

Prior to the initiation of the production process, the fermenter needs to be sterilised. The fermentation media and the fermenter can be sterilised together or separately. The fermenter is sterilised by channelling steam into the vessel via all entries and releasing the steam slowly through air outlet. The jackets or coils of the fermenter are sterilised by heating them with steam. Also the steam pressure is maintained at 15 psi inside vessel for 20 min approximately for thorough sterilisation.

3.7.3 Cultivation in Fermenter

For all the fermentation processes, an active 2 % inoculum raised in an optimised medium is used. The initial pH of the process is 6.5. As the biomass production is initiated, there is uptake of glucose which decreases the pH to between 5.5 and 6.0 in late log phase. Since the optimum pH for sustainable growth of the members of *Sebacinales* is 5.8, there is no requirement for pH control in fermenter systems where the fungal cultures are grown on media containing complex nitrogen sources. The temperature range for the members of *Sebacinales* is in between 20 and 35 °C. However, for optimised growth, the fungal cultures are grown at a temperature of 30 °C. The fungus grows best at lower agitation and low oxygen concentrations (Varma et al. 2001). Thus the cultures are grown at 200 rpm and 20 % working volume (Fig. 3.5).

Fig. 3.5 Production of biomass in bench fermenter (7 litres)

3.7.4 Recovery of Biomass Produced

After the desired biomass is obtained, the production process is terminated. The biomass produced in the fermenter vessel is removed. The produced biomass is then filtered, separating the filtrate from the biomass. After separation the biomass obtained is then formulated by mixing with sterilised magnesium sulphite, talcum powder or vermiculite.

3.8 Production of Nanomycorrhiza

Over the past years extensive research has been carried out on nanocrystalline materials. Various nanoparticles were employed for several purposes (Figs. 3.6 and 3.7). Out of that zinc oxide (ZnO) is the most exploited material. The wide band gap and large excitonic binding energy of ZnO particles correspond to their vast scientific and industrial applications (Wang et al. 2004). The Food and Drug Administration has listed ZnO as a 'generally recognized as safe (GARS)' material (Rajiv et al. 2013). The colloidal solution of ZnO nanorods is utilised as 'nano-fertiliser'. It provides nutrients to plants and also revives the soils natural organic state without the use of chemical fertilisers.

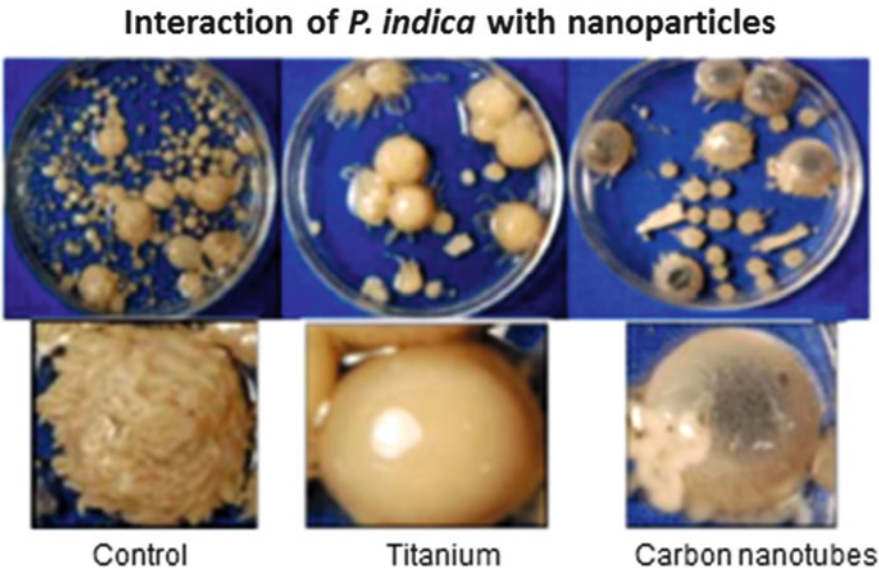

Fig. 3.6 Interaction of *P. indica* with titanium and carbon nanoparticles

Effect of nanoparticles on the biomass of *P. indica*

S. No.	Treatment	Fresh Weight (g/100mL)	Percent increase over control
1	Control	2.98	-
2	Titanium dioxide	4.12	38.25
3	Carbon Nano Tubes	3.86	29.53

Fig. 3.7 Effects of nanoparticles on the biomass of *P. indica* cited from Suman et al. (2010)

3.8.1 Synthesis of Nanoparticles

Nanorods of ZnO are produced following the mechanical-assisted thermal decomposition method (Zhang et al. 2014; Shakti et al. 2014; Noipa et al. 2014). Zinc acetate is used as a precursor for the production of nanorods. For the synthesis the precursor was grinded in a mortar-pestle for 45 min. The homogenous powder is then transferred to an alumina crucible and incubated in an oven. Thus, the process is called as mechanical-assisted thermal decomposition. In case of ZnO nanorods, 2 g of zinc acetate dehydrate was homogenised in mortar-pestle for 45 min and

incubated in furnace at 500 °C for 4 h. The powder thus obtained is washed with distilled water twice and then dried by heating at 100 °C for 8 h.

3.8.2 Preparation of Nanomycorrhiza

For the preparation of nanomycorrhiza, the growth media is augmented with nanoparticles. To the complex media particularly Hill and Kaefer, ZnO nanorods are added at a concentration of 500 ppm. Further to the augmented media, fungal culture is inoculated.

3.8.3 Growth of Nanomycorrhiza

The ZnO-augmented media inoculated with fungal culture are allowed to grow under normal incubation conditions. The biomass is grown at 30 °C in rotary shaker moving at 200 rpm. The fungal culture shows enhanced growth.

3.9 Conclusions

The order *Sebacinales* is a recently described order with a vast diversity of mycorrhizal fungal species. The members of *Sebacinales* show growth-promoting characters. The *Sebacinales* species promote plant growth, induce early flowering and provide resistance against biotic and abiotic stress. Thus it is an excellent biofertiliser and a potential biocontrol agent. Therefore, large-scale production of fungal cultures is much needed for the formulation of biofertilisers.

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References

Ahuja MR (1986) Aspen. In: Evans DA, Sharp WR, Ammirato PJ (eds) Handbook of plant cell culture, Techniques and applications, vol 4. Macmillan, New York, pp 626–651

Basiewicz M, Weiss M, Kogel KH, Langen G, Zorn H, Zuccaro A (2012) Molecular and phenotypic characterization of Sebacina vermifera strains associated with orchids, and the description of Piriformospora williamsii sp. nov. Fungal Biol 116:204–213

Brundrett M (2009) Mycorrhizal associations and other means of nutrition of vascular plants: understanding the global diversity of host plants by resolving conflicting information and developing reliable means of diagnosis. Plant Soil 320:37–77

Deshmukh S, Hückelhoven R, Schä fer P, Imani J, Sharma M et al (2006) The root endophytic fungus Piriformospora indica requires host cell death for proliferation during mutualistic symbiosis with barley. Proc Natl Acad Sci USA 103:18450–18457

Franken P, Requena N, Butehorn B, Krajinski F, Kuhn G, Lapopin L, Mann P, Rhody D, Stommel M (2000) Molecular analysis of the arbuscular mycorrhiza symbiosis. Arch Acker Pft Boden 45:271–286

Gallowey LD, Burgess R (1962) Applied mycology and bacteriology, 3rd edn. Leonard Hill, London, pp. 54–57

Glen M, Tommerup IC, Bougher NL, O'Brien PA (2002) Are Sebacinaceae common and widespread ectomycorrhizal associates of Eucalyptus species in Australian forests? Mycorrhiza 12:243–247

Herrmann S, Munch JC, Buscot F (1998) A gnotobiotic culture system with oak microcuttings to study specific effects of mycobionts on plant morphology before, and in the early phase of, ectomycorrhiza formation by Paxillus involutus and Piloderma croceum. New Phytol 138:203–212

Hibbett DS, Binder M, Bischoff JF, Blackwell M, Cannon PF, Eriksson OE, Huhndorf S, James T, Kirk PM, Lücking R, Lumbsch HT, Lutzoni F, Matheny PB, McLaughlin DJ, Powell MJ, Redhead S, Schoch CL, Spatafora JW, Stalpers JA, Vilgalys R, Aime MC, Aptroot A, Bauer R, Begerow D, Benny GL, Castlebury LA, Crous PW, Dai Y-C, Gams W, Geiser DM, Griffith GW, Gueidan C, Hawksworth DL, Hestmark G, Hosaka K, Humber RA, Hyde KD, Ironside JE, Kõljalg U, Kurtzman CP, Larsson K-H, Lichtwardt R, Longcore J, Miadlikowska J, Miller A, Moncalvo J-M, Mozley-Standridge S, Oberwinkler F, Parmasto E, Reeb V, Rogers JD, Roux C, Ryvarden L, Sampaio JP, Schüßler A, Sugiyama J, Thorn RG, Tibell L, Untereiner WA, Walker C, Wang Z, Weir A, Weiss M, White MM, Winka K, Yao Y-J, Zhang N (2007) A higher-level phylogenetic classification of the fungi. Mycol Res 111:509–547

Hill TW, Kaefer E (2001) Improved protocols for Aspergillus medium: trace elements and minimum medium salt stock solution. Fungal Genet Newsl 48:20–21

Lansing PM, John HP, Donald KA (2005) Microbiology, 6th edn. McGraw-Hill Higher Education, New York

Johnson CN, Stout PR, Broyer RC, Carlton AB (1957) Comparative chlorine requirements of different plant species. Plant Soil 8:337–353

Kaefer E (1977) Meiotic and mitotic recombination in Aspergillus and its chromosomal aberrations. Adv Genet 19:33–131

Kottke I, Guttenberger M, Hampp R, Oberwinkler F (1987) An in vitro method for establishing mycorrhizae on coniferous tree seedlings. Trees 1:191–194

Kottke I, Beiter A, Weiß M, Haug I, Oberwinkler F et al (2003) Heterobasidiomycetes form symbiotic associations with hepatics: Jungermanniales have sebacinoid mycobionts while *Aneura pinguis* (Metzgeriales) is associated with a Tulasnella species. Mycol Res 107:957–968

Kumar V, Sahai V, Bisaria VS (2011) High-density spore production of *Piriformospora indica*, a plant growth promoting endophyte, by optimization of nutritional and cultural parameters. Bioresour Technol 102:3169–3175

Martin JP (1950) Use of acid, rose bengal and streptomycin in the plate method for estimating soil fungi. Soil Sci 69:215–232

Marx DH (1969) The influence of ectotrophic mycorrhizal fungi on the resistance of pine roots to pathogenic infections. I. Antagonism of mycorrhizal fungi to root pathogenic fungi and soil bacteria. Phytopathology 59:153–163

McKendrick SL, Leake JR, Taylor DL, Read DJ (2002) Symbiotic germination and development of the myco-heterotrophic orchid Neottia nidus-avis in nature and its requirement for locally distributed Sebacina spp. New Phytol 154:233–247

Mukerji KG, Mandeep K, Varma A (1998) Mycorrhizosphere microorganisms: screening and evaluation. In: Varma A (ed) Mycorrhiza manual. Springer, Berlin, pp 85–98

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassay with tobacco tissue cultures. Physiol Plant 15:431–487

Noipa K, Rujirawat S, Yimnirun R, Promarak V, Maensiri S (2014) Synthesis, structural, optical and magnetic properties of copper doped ZnO nanorods prepared by a simple direct thermal decomposition route. Appl Phys A 117:927–935

Qiang X, Weiss M, Kogel KH, Schäfer P (2012) Piriformospora indica—a mutualistic basidiomycete with an exceptionally large plant host range. Mol Plant Pathol 13:508–518. doi:10.1111/J.1364-3703.2011.00764

Rajiv P, Rajeshwari S, Venckatesh R (2013) Rambutan peels promoted biomimetic synthesis of bioinspired zinc oxide nanochains for biomedical applications. Spectrochim Acta A Mol Biomol Spectrosc 112:384–387 http://dx.doi.org/10.1016/j.saa.2014.08.022

Selosse M-A, Weiß M, Jany J-L, Tillier A (2002) Communities and populations of sebacinoid basidiomycetes associated with the achlorophyllous orchid Neottia nidus-avis (L.) L.C.M. Rich. and neighbouring tree ectomycorrhizae. Mol Ecol 11:1831–1844

Selosse M-A, Setaro S, Glatard F, Richard F, Urcelay C et al (2007) Sebacinales are common mycorrhizal associates of Ericaceae. New Phytol 174:864–878

Setaro S, Weiß M, Oberwinkler F, Kottke I (2006) Sebacinales form ectendomycorrhizas with Cavendishia nobilis, a member of the Andean clade of Ericaceae, in the mountain rain forest of southern Ecuador. New Phytol 169:355–365

Shakti N, Prakash A, Mandal T, Katiyar M (2014) Processing temperature dependent morphological and optical properties of ZnO nanorods. Mater Sci Semicond Process 20:55–60

Smith S, Read D (1997) Mycorrhizal symbiosis, 2nd edn. Academic, London

Suman RP, Jain VK, Varma A (2010) Role of nanomaterials in symbiotic fungus growth enhancement. Current Science 99:1089–1091

Taylor DL, Bruns TD, Szaro TS, Hodges SA (2003) Divergence in mycorrhizal specialization within Hexalectris spicata (Orchidaceae), a non-photosynthetic desert orchid. Am J Bot 90:1168–1179

Urban A, Weiß M, Bauer R (2003) Ectomycorrhizas involving sebacinoid mycobionts. Mycol Res 107:3–14

Varma A, Singh A, Sudha S, Sahay NS, Sharma J, Roy A, Kumari M, Rana D, Thakran S, Deka D, Bharti K, Franken P, Hurek T, Blechert O, Rexer K-H, Kost G, Hahn A, Hock B, Maier W, Walter M, Strack D, Kranner I (2001) Piriformospora indica: an axenically culturable mycorrhiza-like endosymbiotic fungus. In: Hock B (ed) Mycota, vol 9. Springer, Berlin, pp 123–150

Varma A et al (2013) Piriformospora indica, Soil biology, vol 33. Springer, Berlin. doi:10.1007/978-3-642-33802-1_1

Vezina C, Singh K, Sehgal SN (1965) Sporulation of filamentous fungi in submerged culture. Mycologia 57:722–736

Waller F, Achatz B, Baltruschat H, Fodor J, Becker K et al (2005) The endophytic fungus Piriformospora indica reprograms barley to salt-stress tolerance, disease resistance, and higher yield. Proc Natl Acad Sci USA 102:13386–13391

Wang XD, Ding Y, Summers CJ, Wang ZL (2004) large-scale synthesis of six-nanometer-wide ZnO Nanobelts. J Phys. Chem. B 108:8773

Weiß M, Selosse M-A, Rexer K-H, Urban A, Oberwinkler F (2004) Sebacinales: a hitherto overlooked cosm of heterobasidiomycetes with a broad mycorrhizal potential. Mycol Res 108:1003–1010

Weiß M, Sýkorová Z, Garnica S, Riess K, Martos F (2011) Sebacinales everywhere: previously overlooked ubiquitous fungal endophytes. PLoS One 6:e16793. doi:10.1371/journal.pone.0016793

Yoshida F, Yamane T, Nakamoto K (1973) Fed-batch hydrocarbon fermentations with colloidal emulsion feed. Biotech Bioeng 15:257–270

Zhang J, Greasham R (1999) Chemically defined media for commercial fermentations. Appl Microbiol Biotechnol 51:407–421

Zhang X, Qin J, Xue Y, Yu P, Zhang B, Wang L, Liu R (2014) Effect of aspect ratio and surface defects on the photocatalytic activity of ZnO nanorods. Sci Rep 4:4596

Chapter 4
Biophysical Phenotyping as an Essential Tool for Understanding Host–Microbe Interaction

Avinash P. Ingle, Dnyaneshwar Rathod, Marian Brestic, Hazem M. Kalaji, and Mahendra Rai

Abstract The symbiotic relationship between plant and microbes is one of the most important types of symbiosis among different living forms. Such kind of association helps both the host and the symbiont. Generally, bacteria and fungi are found in association with a variety of plants. It is believed that more than 80 % of plants from different families available on the earth surface have a symbiotic association with arbuscular mycorrhizal fungi. In this context, it is very important to understand the host–microbe interaction, which will elucidate the exact mechanisms involved in the symbiosis.

Plant performance and productivity depend on relationship between host and symbiont. Therefore, the study on comprehensive assessment of biophysical parameters like plant growth, yield, tolerance toward stress, resistance toward various biotic and abiotic agents, plant architecture, physiology, ecology, and the measurement of other quantitative parameters is necessary to know the degree of association.

Chlorophyll *a* fluorescence measurement-based biophysical phenotyping is the description of the behavior and performance of photosystem II (PSII) and photosystem I (PSI) in terms of different structural and functional parameters in photosynthesis. It is mostly studied using JIP test which involves analysis of the chlorophyll *a* fluorescence transient OJIP shown by the photosynthetic plants and algae upon illumination after sample dark adaptation. This technique provides rapid detection and it is easy to handle without damaging the tissues.

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4.1 Introduction

It is believed that photosynthesis is one of the important basis for maintaining all life forms on the earth. It is well known that each and every living form needs energy for its life and growth, but only plants and algae are capable for synthesis of carbohydrates necessary for their growth using solar energy (Khalida et al. 2012). Therefore, photosynthesis by plants and algae is very important because they are primary producers. In the process of photosynthesis, the study of chlorophyll *a* fluorescence is very useful to understand the growth of the plants. It is the most useful marker for the biophysical phenotyping, which provides complete idea about photosynthetic activity. In the dark-adapted photosynthetic plants and algae when illuminated, the intensity of chlorophyll fluorescence kinetics exhibits highly dependent photochemical reactions of photosynthesis (Juneau and Papovic 1999; Kalaji et al. 2014).

Khalida et al. (2012) demonstrated that the absorbed light energy, which is not used for photochemistry of PSII, is automatically dissipated by chlorophyll *a* fluorescence of PSII. The amount of energy degenerated by the fluorescence depends on the competition between the routes of dissipation of energy. The fluorescence can be used to study the electron transport and mechanisms of energy transfer in the process of photosynthesis in green plants and algae.

The study of chlorophyll *a* fluorescence has proved to be of utmost importance because of its complex connection with the frequent processes taking place throughout the energy conversion of light into a stable chemical energy (Papageorgiou and Govindjee 2004). Similarly, kinetics analysis of chlorophyll *a* fluorescence has been a widespread nondestructive method used extensively for the study of oxygenic photosynthetic organisms like higher plants, algae, and cyanobacteria. Moreover, it also provides qualitative as well as quantitative information about photosynthetic activity of green plants and algae (Lazar and Schansker 2009; Kalaji et al. 2012).

Many other fluorescence parameters have been defined in studies that contain samples kept in light showing fluorescence induction, as mentioned earlier. The photosynthetic samples, kept in darkness, are illuminated. Chlorophyll *a* fluorescence intensity shows characteristic changes called fluorescence induction, fluorescence transient, or simply the Kautsky effect (Kautsky and Hirsch 1931). Strasser et al. (2000, 2004) invented a computational tool to analyze chlorophyll *a* fluorescence. It is well known that biophysical parameters are not only useful in the recognition and assessment of the beneficial role of microorganisms on PSII activity (Tsimilli-Michael and Strasser 2002) but also equally helpful in biophysical phenotyping of the photosynthetic apparatus of a plant under stress caused by different factors like temperature, drought, and light intensity (Rathod et al. 2011; Brestic et al. 2014).

This chapter is focused on various biophysical parameters and the role of biomarkers like chlorophyll *a* fluorescence analysis for the understanding of host–symbiont interactions. Further, we have also discussed about advanced

technological solutions available for the analysis of chlorophyll *a* fluorescence, which helps in the study of biophysical phenotyping in a simple, rapid, and ecofriendly manner.

4.2 Chlorophyll *a* Fluorescence as a Biomarker

Nowadays, analysis of chlorophyll *a* fluorescence has become the prime choice of physiologists and botanists and is known as an important biomarker in this context (Maxwell and Jonhson 2000). Although, chlorophyll *a* fluorescence corresponds to a very small fraction of the dissipated energy from the photosynthetic apparatus, it is widely accepted to offer an access to the understanding of its structure and function (Strasser et al. 2004).

Chlorophyll *a* fluorescence, which results from excitation of chlorophyll *a* molecule, can be used as a biophysical marker in many applications, such as evaluation of stress (microbial and environmental), in understanding host–symbiont interaction and also for the effects on PSII system (Strasser et al. 2000; Epitalawage et al. 2003; Goltsev et al. 2005). Usually, light energy is absorbed by different pigments of chloroplasts such as chlorophyll, carotenoids, and others present in the photosynthetic antenna in the thylakoid membranes of green plants (Strasser et al. 2000, 2004; Govindjee 2004). After a photon is absorbed, due to excitation chlorophyll *a* molecule is transferred to its lowest singlet excited state, for which three internal pathways exist: (i) fluorescence, in which the molecule returns to the ground state with the emission of radiation; (ii) internal conversion, in which the energy of the molecule is converted into vibrational energy; and (iii) intersystem crossing, in which the singlet state is converted to the triplet state. Hence, chlorophyll *a* fluorescence analysis is considered as an important tool for the biologists studying photosynthesis and other fields related to biophysics, biochemistry, and physiology of green plants (Misra et al. 2012).

Marwood et al. (2000, 2001) demonstrated the importance of chlorophyll *a* fluorescence analysis in the study of plants exposed to polycyclic aromatic hydrocarbons (PAHs) and photo-modified PAH mixtures. In another study, Schreiber et al. (1986) proposed that pulse amplitude-modulated chlorophyll *a* fluorescence was found to be rapid tool for measuring photosynthetic electron transport in plants in vivo. Similarly, chlorophyll *a* fluorescence analysis is also very useful, fast, ecofriendly, and economically viable biomarker to assess the toxicity caused by PSII inhibitors and also provides some indication regarding the mode of action of a given xenobiotic (Fai et al. 2007). In addition, the analysis of chlorophyll *a* fluorescence is considered to be authentic evidence of the plant health under abiotic stress. Gururani et al. (2015) successfully evaluated the influence of cold stress on the photosynthetic apparatus of transgenic turf grass (*Zoysia japonica*) through chlorophyll *a* fluorescence analysis. From the analysis of OJIP test, remarkable difference was reported in the physiological status among plants under cold stress and control plants. Finally, they proposed that chlorophyll *a* fluorescence analysis

would be an efficient tool which can provide relevant information about the physiology of plants growing under abiotic stress.

But apart from these applications, chlorophyll *a* fluorescence can also be utilized as an efficient biomarker in understanding the host–symbiont interactions. Recently, Rozpadek et al. (2015) studied the symbiotic interaction of endophytic fungus *Epichloe typhina* with its host plant *Dactylis glomerata* (an orchid grass), particularly effects of the endophytes on photosynthesis apparatus. Certain markers such as chlorophyll *a* fluorescence, gas exchange, immunoblotting, spectrophotometric measurements, etc., were selected for assessment of photosynthetic performance, changes in pigment content, and mechanisms associated with light harvesting, carbon assimilation, and energy distribution in host plant. The result obtained from analysis of chlorophyll *a* fluorescence provides evidence of increase in chlorophyll contents (Rozpadek et al. 2015).

It is well known that arbuscular mycorrhizal fungi (AMF) as symbiont play a crucial role in the supply of plant nutrition by supplying mineral nutrients, particularly inorganic phosphate, and also constitute an important carbon sink. In this context, Adolfsson et al. (2015) studied the interaction of *Medicago truncatula* plants grown with *Rhizophagus irregularis*, a type of AM fungi. Here, analysis of chlorophyll fluorescence indicated that AM symbiosis did not alter the photosynthetic activity per leaf area. Thus, an increase in photosynthetic production was achieved due to increased surface for sunlight absorption.

4.3 Discovery of Handy PEA

The Handy PEA (Plant Efficiency Analyser) instrument was launched in 2001, succeeding the PEA instrument, Hansatech Instruments Ltd, King's Lynn, Norfolk, UK. The Handy PEA instrument, also known as chlorophyll fluorimeter or stress meter, is supplied with a Windows data transfer and analysis software package (Strasser et al. 2007). For data transfer and analysis, the computer-based Biolyzer program (R. Maldonado-Rodriguez, Laboratory of Bioenergetics, University of Geneva) can be used. The Handy PEA consists of a compact, light-weight control unit encapsulating sophisticated electronics, which provides the high time resolution essential in performing measurements of fast chlorophyll fluorescence induction kinetics, and it is supplied with sensor head (Fig. 4.1).

The working principle of this instrument is based on the collection of chlorophyll fluorescence signal by the sensor head during measurements, and further these signals are digitized using a fast specialized analogue/digital converter situated within the Handy PEA control unit. The instrument facilitates digitization of chlorophyll fluorescence signal collected by sensor head at different rates depending upon the various phases of the induction kinetic. Initially, data is sampled at 10 μs (micro seconds) intervals for the first 300 μs. Handy PEA chlorophyll fluorimeter is a simple instrument and easy to operate. Moreover, it has the capacity to store up to six user-defined protocols for different field applications. About 1000

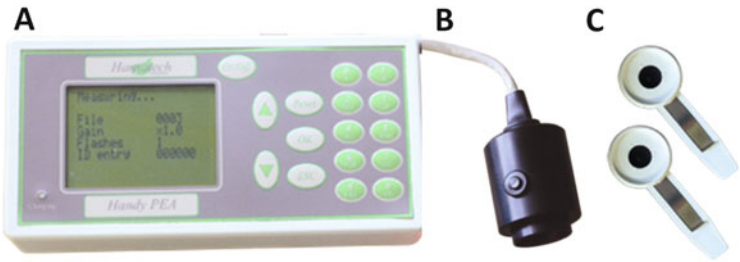

Fig. 4.1 Handy PEA instrument and their accessories, where (**a**) Handy PEA instrument, (**b**) Holder, (**c**) Leaf clip

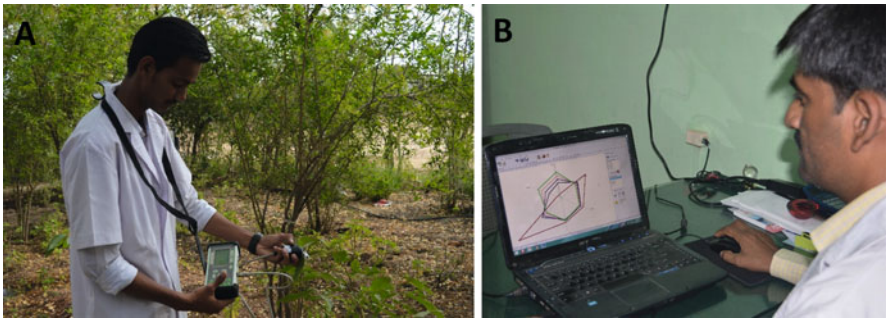

Fig. 4.2 (**a**) Measurement of chlorophyll *a* fluorescence using Handy PEA. (**b**) Analysis of chlorophyll *a* fluorescence data using Biolyzer program

recordings can be easily saved in the memory of Handy PEA chlorophyll fluorimeter. Data recorded in the instrument may be viewed in numerical format and can be analyzed after transferring to the computer using PEA Plus software where it may be viewed graphically or exported to external software packages for further statistical analysis (http://www.hansatech-instruments.com/products/introduction-to-chlorophyll-fluorescence/continuous-excitation-chlorophyll-fluorescence/handy-pea). Figure 4.2 represents the measurement of chlorophyll *a* fluorescence using Handy PEA and analysis of data using Biolyzer program.

4.4 Measurement of Different Fluorescence Parameters

For the measurement of chlorophyll *a* fluorescence, Handy PEA facilitates measurement of various key fluorescence parameters. Detailed analysis of these parameters using either PEA Plus or Biolyzer program gave an idea about the photosynthetic machinery of the green plants and algae.

Usually, the following basic measured and calculated parameters can be seen on the screen of Handy PEA:

Fo It is the initial fluorescence level when plastoquinone electron acceptor pool (Qa) is fully oxidized. The program (algorithm) is used to determine the line of best fit through the initial data point recorded at the onset of illumination. This line of best fit is extrapolated to time zero (start of illumination) to determine Fo. The value obtained for Fo of different specimens at the same level may be used for comparison. The Fo level is thought to represent emission by excited chlorophyll a molecule in the PS II.

Fm It is the maximum fluorescence level obtained for the same light intensity. It will only be correct if the light intensity is fully saturating for the plant and the electron acceptor Qa is fully reduced. In order to compare the absolute value of the fluorescence signal, which is proportional to the excitation intensity, it is important that comparison is only made between measurements at the same intensity.

Fv It is a variable component of fluorescence. It can be calculated by subtractions of Fo from the Fm values (i.e., Fm – Fo).

Fv/Fm It is the ratio of variable fluorescence divided by the maximum fluorescence. It has been shown to be proportional to the quantum yield of net photosynthesis. This ratio is independent of leaf area. But it requires absolute values of Fo and Fm.

TFm It is the time at which the maximum fluorescence occurs.

Area The area above the fluorescence curve between Fo and Fm is proportional to the pole size of electron acceptor Qa on the reducing side of photosystem II. The area measurement is very useful parameter as it highlights any change in the shape of induction kinetic between Fo and Fm which would not be evident from the other parameters, Fo, Fm, and Fv/Fm.

PI It is the performance index of PSII. The performance index is essentially an indicator of sample vitality. It is an overall expression indicating a kind of internal force of the sample to resist constraints from outside. It is a force in the same way redox potential in a mixture of redox couples is a force. Exactly the PI is a force if used on log scale. Therefore we say:

$$\log PI = \text{Driving Force DF}$$

The PI or performance index is derived according to the Nernst equation. It is the equation which describes the forces of redox reactions and generally movements of Gibbs free Energy in biochemical systems.

4.5 What Is JIP Test?

The fluorescence transient O-J-I-P is measured in terms of JIP test. The term JIP test was adopted from the work of Prof. Reto Strasser and his colleagues at the laboratory of Bioenergetics, University of Geneva, Switzerland (Fig. 4.3). The fluorescent parameter resulting from the relative amplitude of extremes of the fluorescence signals, e.g., Fo (the minimal fluorescence intensity measured at 50 μs) and Fp (the maximal fluorescence intensity). The OJIP analysis shows intermediate step in the fluorescence induction transient such as the *J* step at 2 ms and *I* step at 30 ms. Thus, in these circumstances the fluorescence induction kinetics can be characterized as *O-J-I-P*.

The Handy PEA software uses the fluorescence value at these time marks to derive a series of many further parameters calculated per reaction center (RC) or cross section (CS) and also the PI (performance index) which is calculated as

$$(\mathrm{RC/ABS}) \times (\phi\mathrm{Po}/(1-\phi\mathrm{Po})) \times (\mathrm{o}/(1-\mathrm{o}))$$

where

RC is the reaction center,
ABS is the absorption flux,
ϕPo is the maximal quantum yield for primary photochemistry,
and o is the quantum yield for electron transport (Kalaji et al. 2011).

The JIP test generally provides a quantitative analysis of the in vivo vitality behavior and performance of pigment system II (PSII), that is, a quantitative description of the biophysical phenotype macro-state, by accessing the different microstate-functional building blocks (Strasser et al. 2007). The JIP test is also considered important tool for the analysis of fast fluorescence kinetics O-J-I-P exhibited by all oxygenic photosynthetic organisms upon illumination, based on a

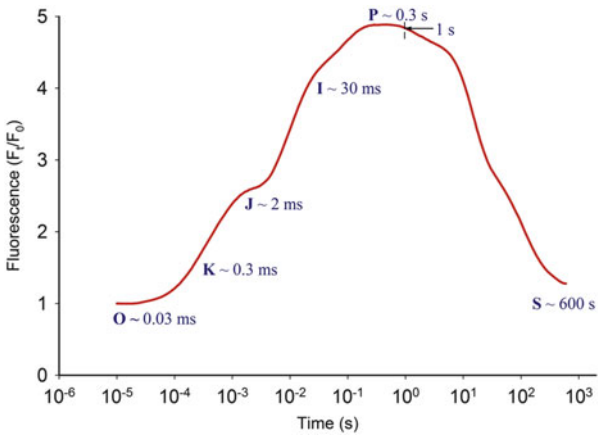

Fig. 4.3 A standard OJIP chlorophyll fluorescence induction curve with main steps and their appearance time

simple model and the theory of energy fluxes in biomembranes (Rathod et al. 2011). It is well reported that biophysical tools are very important in the recognition and evaluation of beneficial role of microorganisms on PSII activity (Tsimilli-Michael and Strasser 2002). In addition, it is also important in biophysical phenotyping of the photosynthetic apparatus of a plant under any kind of stress, such as stress caused by drought, light, and temperature (Zivcak et al. 2015).

4.5.1 *JIP Test Is the Rapid and Sensitive Technique in Assessing the Efficacy of Any Microorganism on Growth Promotion of Plants*

Rai et al. (2008) studied the effect of various AMF in plant growth promotion of maize (*Zea mays*) through the analysis of chlorophyll *a* fluorescence and JIP test. In their experiment, they inoculated the maize plants with AMF, *P. indica,* and *G. mosseae* separately and also with mixed culture of *P. indica, G. mosseae*, and *G. caledonium*. The non-inoculated plants (without AMF) were maintained as control. Chlorophyll *a* fluorescence data were measured at intervals of 7 days up to 60 days. It was observed that the various JIP test parameters are highly affected in both treated and non-treated plants. The increase in the yield for electron flow ($\phi Eo = \phi Po * \psi o$) was reported in plants inoculated with *P. indica* and mixed culture. Similarly, electron transport per trapping center (ψo) was remarkably influenced by the treatment of AMF. Plants treated with *P. indica* and mixed culture showed relatively higher quantum yield compared to non-inoculated plants. Moreover, absorption per reaction center was also reported higher in plants treated with AMF as compared to controls. The morphological characters like height of the plants and percentage colonization in the roots also strengthen the plant growth promotion due to treatment of AMF. Therefore, the authors concluded that the JIP test can be used as a sensitive and rapid tool for determination of efficiency of AMF in host plants.

Rathod et al. (2011) also reported that JIP test was found to be a rapid and nondestructive technique and can be used for the evaluation of the effect of mycorrhizal on growth of plants. However, for higher plants and algae, chlorophyll *a* fluorescence induction curve measured under continuous light has a fast (within a second) increasing phase and a slow (within a few minutes) decreasing phase. Further, light-exposed samples also exhibit fluorescence changes, upon change of wavelength or intensity of light (Strasser et al. 2004). After the light is turned off and a sufficiently long dark period is given (e.g., 15–30 min to 1 h), the original fluorescence induction kinetics are observed again when the same light is turned back on. This transient has inflection points for a history of the nomenclature used for fluorescence transient curves): the fast phase is labeled as OJIP, where O is the origin, the first measured minimal level, J and I are intermediate levels, and P is the peak.

4.6 Biophysical Parameters

Different biophysical parameters, such as grain yield, plant height, plant age, crown diameter, diameter at breast height, basal area, tree density, biomass, root length, number of roots, number of leaves, size of leaves, leaf area index, chlorophyll-content index, etc., have been considered as important morphological parameters in the context of plant growth. In particular, such biophysical parameters always acts as natural indicators directly or indirectly toward proper plant growth, vegetation in agriculture and forest, environmental changes, management, and weather forecasting (Monteiro et al. 2012; Avtar et al. 2013; Das et al. 2015). Also, study of various biophysical parameters of plants like height, age, crown diameter, plant diameter basal area, tree density, and biomass is considered to be essential for the management practices in plants such as location determination, harvesting, replantation, yield estimation, and carbon accumulation (Avtar et al. 2013).

Considering the importance of biophysical parameters, Jirali et al. (2008) carried out a field experiment on two types of turmeric genotypes (i.e., Amalapuram and Rajapuri) at the University of Agricultural Sciences, Dharwad, Karnataka, India, and studied the effect of plant growth regulators on biophysical, biochemical parameters, yield, and quality. It was reported that the treatment of various plant growth regulators such as chlormequat (1000 ppm), Cytozyme (2000 ppm), and Miraculan (2000 ppm) was very effective. Moreover, these plant growth regulators significantly increased the important biophysical parameters, photosynthetic rate and transpiration rate, and total chlorophyll content.

In another study, Singh et al. (2015) demonstrated that levels of spacing between cotton plants may affect the biophysical and biochemical parameters. Further, they reported that different levels of plant spacing, i.e., 40, 50, and 60 cm, with a consistent row width of 210 cm increased the various biophysical parameters like relative water content, leaf area index, and biochemical parameters like chlorophyll content of cotton crop during different stages of crop growth together with yield and yield components.

Various methods have been proposed and developed for characterization and monitoring of different biophysical parameters. Among those, remote sensing satellite method is becoming more popular. The remote sensing technology is considered as easy approach to study vegetation cover and associated temporal changes using satellite at a regional level. Biophysical parameters recorded from remote sensing satellites can be utilized for several applications such as forestry, agriculture, land management, as well as weather forecasting. According to Das et al. (2015), spatial and regional information about the biophysical parameters like chlorophyll and leaf-area index which are considered as indicators for crop condition is very useful to guide farmers, planners, and managers toward sustainable management of their agricultural resources. So in this context, remote sensing method will be an efficient method for spatial characterization of crop biophysical parameters with the aim of having a wider perspective for ecological information and modeling.

4.7 Biolyzer Program: Unique Analysis and Graphical Representation

Biolyzer is a software-based computer program specifically designed for the analysis of chlorophyll fluorescence data obtained with the help of Handy PEA (i.e., fluorometer equipment). Biolyzer is a computerized program, which can be used for the evaluation of efficiency of photosynthesis in plants. The analysis of photosynthetic efficiency gives the idea about the various parameters like stress in plants, algae, and cyanobacteria which affects the plant growth. According to the experts, Biolyzer program is very useful for classification of fluorescence data recorded using Handy PEA in order to determine plant groups that show different photosynthetic responses. The determination of various factors responsible for low photosynthesis in plants can help to prevent damage caused by such factors, before it is too late (http://www.fluoromatics.com/biolyzer_software-1.php).

Biolyzer program has been specifically built for the calculation of several parameters of JIP tests, and two important foundations, i.e., speed optimization and robust algorithms, were used as a basis for this software. It was reported to be the most innovative and rapid technique for the analysis of chlorophyll *a* fluorescence (OJIP signals) measured for plants, green algae, and cyanobacteria. To date, various versions of Biolyzer program have been developed, but Biolyzer® 5.0 software provides full calculations of the JIP Test, and hence, it has been recognised as the best and the faster ever released version. In addition, it has many useful graphical and diagram tools for extracting more and more information out of your fluorescence data (http://www.fluoromatics.com/biolyzer_software-1.php).

As mentioned above, Biolyzer is not only useful for the calculations of simple JIP test parameters but also provides unique analysis and colorful graphical displays of professional standard. Also, preparation of multidimensional classy plots from large sets of data and their components is possible. Moreover, it facilitates representation of raw and processed data in a leading edge graphical format, which allows the user to intuitively discover, analyze, and interpret novel relationships within the recorded data. Most important point is that there is no need of prior knowledge of statistical methods or clustering techniques; without knowledge of such methods, analysis of complex data in a much simpler way can be possible. Figure 4.4 shows the different graphical ways of representation during the analysis of chlorophyll *a* fluorescence data using Biolyzer program.

4.8 Understanding Host–Symbiont Interaction

Symbiosis in a broad sense can be defined as the "living together in an intimate association of two or more dissimilar organisms." Symbiosis is a relationship in which both organisms benefit simultaneously. There are various kinds of symbiotic associations possible between two different biological systems among the plants, animals, and microorganisms. In this chapter, we focus only on the important plant–

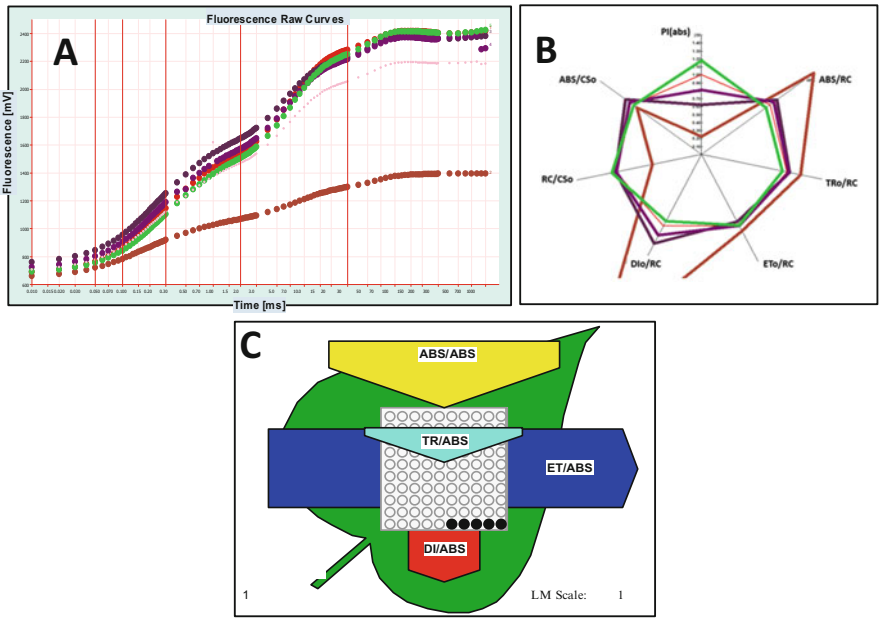

Fig. 4.4 Different graphical ways of representation using Biolyzer program. (**a**) Fluorescence plot, (**b**) Radar plot, (**c**) Pipeline leaf model

microbe symbiotic interactions in general and plant–fungi relationship in particular. In actual, plant–microbe interaction is a complex relationship that can have various beneficial impacts on both the communities. The common examples for plant–microbe interaction include nitrogen fixation by legumes which is a consequence of microbes that fix nitrogen and plants that supply simple carbons. Similarly, in case of symbiosis between plants and fungi, they establish a relationship in which the plant provides nutrients and the fungi provide components to deter predation and allow for greater drought tolerance (Wells and Varel 2011).

Generally, in plant–microbe interactions, two symbiotic systems have been thoroughly studied for many years: arbuscular mycorrhizal fungal (AMF) symbiosis (Fig. 4.5) and root nodule (RN) symbiosis. Among these two systems, the plant–AMF symbiosis is considered as the most widespread interaction between plants and microbes, as far as the phylogeny and ecology is concerned. It was also demonstrated that more than 80 % of plant families available on the earth surface have a symbiotic association with AM fungi which belong to the Glomeromycota group of fungi. According to Parniske (2008), the origin of AMF symbiosis with plant is very old and it is thought to be in the early Devonian period (i.e., approximately 400 million years ago). Therefore, AMF symbiosis is also called the mother of plant root endosymbiosis. However, RN symbiosis involves morphogenesis and is formed by communication between plants and nitrogen-fixing bacteria.

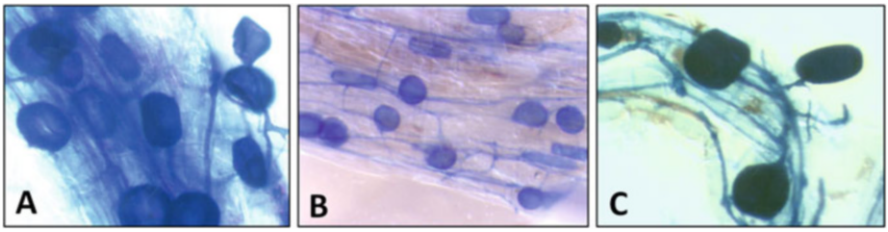

Fig. 4.5 Spores of arbuscular mycorrhizal fungi: (**a**) *Glomus intraradices*, (**b**) *Glomus mosseae*, (**c**) *Piriformospora indica* [Adapted from Rathod et al. (2011) (open access journal)]

Zuccaro et al. (2014) reviewed that a wide range of beneficial fungi are commonly associated with different plants in their roots, in which fungi help the plants for mineral nutrient uptake, whereas in exchange of that fungi get carbohydrates and other organic metabolites. Moreover, it was believed that such kind of associations plays a key role in shaping the terrestrial ecosystems and it also promoted the evolution of land plants. For the establishment of compatible association with their host, root-associated fungi have evolved their strategies of colonization with different functional, morphological, and genomic specializations. These fungi mainly include obligate biotrophic AMF and facultative biotrophic ectomycorrhizal (ECM) interactions but are not restricted to these well-characterized symbiosis. Emergent evidences are recorded in case of root endophytic associations, but unfortunately, such associations are overlooked due to their inconspicuous nature. However, such associations may be symbiotic in nature and represent important players in natural and managed environments. Moreover, some of the recent studies in the field of biology and genomics of root associations revealed interesting insight into the phenotypic and trophic plasticity of these fungi and underlined genomic traits associated with biotrophy and saprotrophy.

For understanding the actual host–symbiont relationship, Rey and Schornack (2013) comparatively studied the pathogenic and symbiotic lifestyles of plant and associated fungi (viz., *Phytophthora* species and AMF). According to them, interaction by both *Phytophthora* species and AMF follows similar developmental processes of identification, plant cell penetration, and redifferentiation of the host cells to establish intracellular interfaces for nutrient and information exchange. Petutschnig et al. (2010) demonstrated that the chitin oligomers of fungal origin mostly act as potent inducers of plant immunity and also played important role in the activation of symbiosis-related signaling (Genre et al. 2013). Furthermore, effector proteins, which are considered as hallmarks of plant pathogens and are responsible for the suppression of defense mechanism and reprogramming of the host plant, were reported in mycorrhizal fungi which on the contrary promote symbiosis. (Kloppholz et al. 2011; Plett et al. 2011).

Recently, Ogar et al. (2015) demonstrated the effect of Zn–Pb contaminations in *Hieracium pilosella* and *Medicago sativa* plants inoculated with AMF using JIP test analysis. According to them, plants inoculated with AMF and contaminated with

Zn and Pb showed significant increase of Fv/Fm parameter for *Hieracium pilosella* as compared with non-inoculated plants, whereas *Medicago sativa* did not exhibit any changes regarding Fv/Fm parameters. Increase in the Fv/Fm parameter suggested the positive effect of AMF inoculation on photosynthesis functioning. Similarly, different measurements were recorded for other parameters used in JIP test such as (ABS/RC), trapping (TR0/RC), and reduction of end acceptors at PSI electron acceptor side (ET0/RC) and the dissipated energy flux (DI0/RC) for both controls (non-inoculated) and plants inoculated with AMF which confirms the symbiotic role of AMF in *H. pilosella* and *M. sativa* contaminated with Zn and Pb.

Similarly, a case study was carried out at the laboratory of Prof. Mahendra Rai (data unpublished) on chickpea (*Cicer arietinum*) plants, in which the role of different endophytes (*G. mosseae*, *P. indica*, and *G. caledonium*) was studied on growth of chickpea subjected to cadmium (Cd) stress. The biophysical parameters such as height of plants, number of leaves, etc., showed remarkable difference as compared to control plants (non-inoculated with AMF). It was reported that the plants subjected to Cd stress and inoculated with *G. Caledonium* showed maximum average height of 28.12 cm (Fig. 4.6c) followed by plants treated with *P. indica* (26.00 cm) (Fig. 4.6d), *G. mosseae* (24.12 cm) (Fig. 4.6b), and control (non-inoculated) plants (14.25 cm) (Fig 4.6a), respectively, after 28 days of sowing. Moreover, the chlorophyll *a* fluorescence data recorded for JIP test analysis also showed increased performance index (PI) and decrease in dissipation (Dio/RC) in plants inoculated with AMF as compared to control plants, which confirmed that AMF played important role in the elevation of Cd stress in chickpea plants.

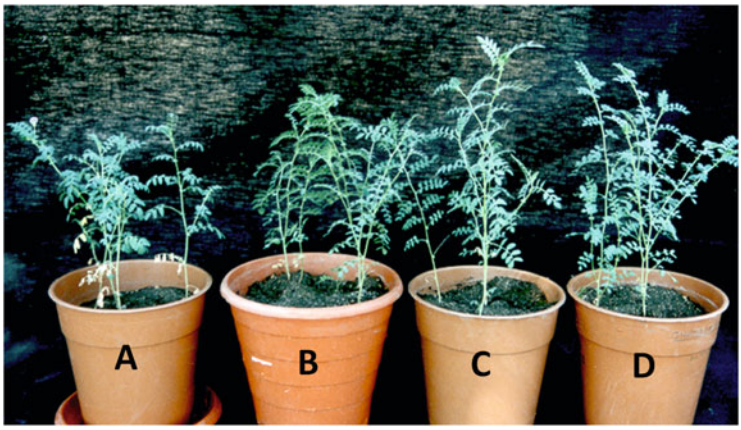

Fig. 4.6 Chickpea plants subjected to Cd stress and various treatments. (**a**) Control plants (without AMF), (**b**) plants inoculated with *G. mosseae*, (**c**) plants inoculated with *G. caledonium*, and (**d**) plants inoculated with *P. indica*

4.9 Conclusions

It is believed that symbiotic relationship between two different living organisms plays a key role in shaping the terrestrial ecosystems. It also helps in the promotion of evolution of land plants. Moreover, the study of various biophysical parameters and biophysical phenotyping, i.e., the measurement of chlorophyll *a* fluorescence using a compact Handy PEA device and their compressive analysis with the help of computer-based software program (Biolyzer), can be used for the evaluation of the vitality, growth promotion of plants, and also for understanding the host–symbiont interactions. It can be concluded that microbial symbiotic partner of plant exerts beneficial role on photosynthetic apparatus (PSII) of plant. Further, JIP test used for biophysical phenotyping is a rapid and nondestructive technique that can be applied for evaluating the effectiveness of symbiotic interaction among plants and fungi like AMF. It can also be applied for the selection of potential symbionts for growth promotion of plants.

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References

Adolfsson L, Solymosi K, Andersson MX, Keresztes A, Uddling J, Schoefs B, Spetea C (2015) Mycorrhiza symbiosis increases the surface for sunlight capture in *Medicago truncatula* for better photosynthetic production. PLoS One 10(1):e0115314. doi:10.1371/journal.pone.0115314

Avtar R, Takeuchi W, Sawada H (2013) Monitoring of biophysical parameters of cashew plants in Cambodia using ALOS/PALSAR data. Environ Monit Assess 185:2023–2037

Brestic M, Zivcak M, Olsovska K, Hong-Bo S, Kalaji MH, Suleyman I (2014) Reduced glutamine synthetase activity plays a role in control of photosynthetic responses to high light in barley leaves. Plant Physiol Biochem 81:74–83

Das PK, Kameswara-Rao SVC, Sravan-Kumar G, Seshasai MVR (2015) Retrieval of biophysical parameters for cotton and rice crops using Resourcesat-2 AWiFS data in Nalgonda district, Andhra Pradesh, India. J Spatial Sci 60:353–363

Epitalawage N, Eggenberg P, Strasser RJ (2003) Use of fast chlorophyll *a* fluorescence technique in detecting drought and salinity tolerant chickpea (*Cicer arietinum* L.) varieties. Archs Sci Genève 56:79–93

Fai PB, Grant A, Reid B (2007) Chlorophyll *a* fluorescence as a biomarker for rapid toxicity assessment. Environ Toxicol Chem 26:1520–1531

Genre A, Chabaud M, Balzergue C, Puech-Pages V, Novero M, Rey T, Fournier J, Rochange S, Becard G, Bonfante P, Barker DG (2013) Short-chain chitin oligomers from arbuscular mycorrhizal fungi trigger nuclear $Ca(2^{+})$ spiking in *Medicago truncatula* roots and their production is enhanced by strigolactone. New Phytol 198:190–202

Goltsev V, Chernev P, Zaharieva I, Lambrev P, Strasser RJ (2005) Kinetics of delayed chlorophyll *a* fluorescence registered in milliseconds time range. Photosynth Res 84:209–215

Govindjee (2004) Chlorophyll *a* fluorescence: a bit of basics and history. In: Papageorgiou GC, Govindjee (eds) Chlorophyll *a* fluorescence: a signature of photosynthesis, Advances in photosynthesis and respiration, vol 19. Springer, Dordrecht, pp 1–41

Gururani MA, Venkatesh J, Ganesan M, Strasser RJ, Han Y, Kim J-I, Hyo-Yeon Lee HY, Song PS (2015) *In vivo* assessment of cold tolerance through Chlorophyll *a* fluorescence in transgenic Zoysiagrass expressing mutant phytochrome A. PLoS One 10(5):e0127200. doi:10.1371/journal.pone.0127200

Jirali DI, Hiremath SM, Chetti MB, Patil SA (2008) Biophysical, biochemical, parameters, yield and quality attributes affected by plant growth regulators in turmeric. Plant Arch 8:275–278

Juneau P, Papovic R (1999) Evidence for the rapid phytotoxicity and environmental stress evaluation using the PAM fluorometric method: importance and future application. Ecotoxicology 8:449–455

Kalaji HM, Schansker G, Ladle RJ, Goltsev V, Bosa K, Allakhverdiev SI, Brestic M, Bussotti F, Calatayud A, Dąbrowski P, Elsheery NI, Ferroni L, Guidi L, Hogewoning SW, Jajoo A, Misra AN, Nebauer SG, Pancaldi S, Penella C, Poli D, Pollastrini M, Romanowska-Duda ZB, Rutkowska B, Serôdio J, Suresh K, Szulc W, Tambussi E, Yanniccari M, Zivcak M (2014) Frequently asked questions about in vivo chlorophyll fluorescence: practical issues. Photosynth Res 122:121–158

Kalaji MH, Goltsev V, Bosa K, Allakverdiev SI, Strasser RJ, Govindjee (2012) Experimental in vivo measurements of light emission in plants: a perspective dedicated to David Walker. Photosynth Res 114:69–96

Kalaji MH, Govindjee BK, Kościelniak J, Żuk-Gołaszewska K (2011) Effects of salt stress on Photosystem II efficiency and CO_2 assimilation of two Syrian barley landraces. Environ Exp Bot 73:64–72

Kautsky H, Hirsch A (1931) Neue Versuche zur Kohlensäureassimilation. Naturwissenschaften 19:964. doi:10.1007/BF01516164

Khalida Z, Youcef A, Zitouni B, Mohammed Z, Radovan P (2012) Use of chlorophyll fluorescence to evaluate the effect of chromium on activity photosystem II at the alga *Scenedesmus obliquus*. Int J Res Rev Appl Sci 12:304–312

Kloppholz S, Kuhn H, Requena N (2011) A secreted fungal effector of *Glomus intraradices* promotes symbiotic biotrophy. Curr Biol 21:1204–1209

Lazar D, Schansker G (2009) Models of chlorophyll *a* fluorescence transients. In: Laisk A, Nedbal L, Govindjee (eds) Photosynthesis in silico: understanding complexity from molecules to ecosystems, Advances in photosynthesis and respiration, vol 29. Springer, Dordrecht, pp 85–123

Marwood CA, Smith REH, Furgal JA, Charlton MN, Solomon KR, Greenberg BM (2000) Photoinhibition of natural phytoplankton assemblages in Lake Erie exposed to solar ultraviolet radiation. Can J Aquat Fish Sci 57:371–379

Marwood CA, Solomon KR, Greenberg BM (2001) Chlorophyll fluorescence as a bioindicator of effects on growth in aquatic macrophytes from mixtures of polycyclic aromatic hydrocarbons. Environ Toxicol Chem 20:890–898

Maxwell K, Jonhson GN (2000) Chlorophyll fluorescence a practical guide. J Exp Bot 51:659–668

Misra AN, Misra M, Singh R (2012) Chlorophyll Fluorescence in Plant Biology. In: Misra AN (ed) Biophysics. InTech Open Science Publisher, Croatia Available at http://www.intechopen.com/books/biophysics/chlorophyll-fluorescence-in-plant-biology

Monteiro PFC, Filho RA, Xavier AC, Monteiro ROC (2012) Assessing biophysical variable parameters of bean crop with hyperspectral measurements. Sci Agric 69:87–94

Ogar A, Sobczyk L, Turnau K (2015) Effect of combined microbes on plant tolerance to Zn-Pb contaminations. Environ Sci Pollut Res 22:19142–19156

Papageorgiou GC, Govindjee (2004) Chlorophyll *a* fluorescence: a signature of photosynthesis, advances in photosynthesis and respiration, vol 19. Springer, Dordrecht, pp. 1–42

Parniske M (2008) Arbuscular mycorrhiza: the mother of plant root endosymbioses. Nat Rev Microbiol 6: 763–775

Petutschnig EK, Jones AM, Serazetdinova L, Lipka U, Lipka V (2010) The lysin motif receptor-like kinase (LysM-RLK) CERK1 is a major chitin-binding protein in *Arabidopsis thaliana* and subject to chitin-induced phosphorylation. J Biol Chem 2010(285):28902–28911

Plett JM, Kemppainen M, Kale SD, Kohler A, Legue V, Brun A, Tyler BM, Pardo AG, Martin F (2011) A secreted effector protein of *Laccaria bicolor* is required for symbiosis development. Curr Biol 21:1197–1203

Rai MK, Shende S, Strasser RJ (2008) JIP test for fast fluorescence transients as a rapid and sensitive technique in assessing the effectiveness of arbuscular mycorrhizal fungi in *Zea mays*: Analysis of chlorophyll *a* fluorescence. Plant Biosys 142:191–198

Rathod DP, Brestic M, Shao HB (2011) Chlorophyll *a* fluorescence determines the drought resistance capabilities in two varieties of Mycorrhized and non-mycorrhized *Glycine max* Linn. Afr J Microbiol Res 5:4197–4206

Rey T, Schornack S (2013) Interactions of beneficial and detrimental root colonizing filamentous microbes with plant hosts. Genome Biol 14:121. doi:10.1186/gb-2013-14-6-121

Rozpadek P, Wezowicz K, Nosek M, Wazny R, Tokarz K, Lembicz M, Miszalski Z, Turnau K (2015) The fungal endophyte *Epichloe typhina* improves photosynthesis efficiency of its host orchard grass (*Dactylis glomerata*). Planta 242:1025–1035

Schreiber U, Schliwa U, Bilber B (1986) Continuous recording of photochemical and non-photochemical chlorophyll fluorescence quenching with a new type of modulation fluorometer. Photosynth Res 10:51–62

Singh DB, Malhi RKM, Kiran GS (2015) Assessing the impact of agronomic spacing conditions on biophysical and biochemical parameters along with yield and yield components in cotton. Int J Agr Agri Res 6:36–44

Strasser RJ, Srivastava A, Tsimilli-Michael M (2000) The fluorescence transient as a tool to characterize and screen photosynthetic samples. In: Yunus M, Pathre U, Mohanty P (eds) Probing photosynthesis: mechanism, regulation and adaptation. Taylor and Francis, London, pp 443–480

Strasser RJ, Tsimilli-Michael M, Dangre D, Rai M (2007) Biophysical phenomics reveals functional building blocks of plant systems biology: A case study for the evaluation of the impact of mycorrhization with *Piriformospora indica*. In: Varma A, Oelmüller R (eds) Soil biology: advanced techniques in soil microbiology. Springer, Berlin, pp 319–341

Strasser RJ, Tsimilli-Michael M, Srivastava A (2004) Analysis of the chlorophyll fluorescence transient. In: Papageorgiou GC, Govindjee (eds) Chlorophyll fluorescence: a signature of photosynthesis, Advances in photosynthesis and respiration, vol 19. Springer, Dordrecht, pp 321–362

Tsimilli-Michael M, Strasser RJ (2002) Mycorrhization as a stress adaptation procedure. In: Gianinazi S, Schüepp H, Barea JM, Haselwandter K (eds) Mycorrhizal technology in agriculture. Birkhäuser Verlag, Switzerland, pp 199–210

Wells J, Varel V (2011) Symbiosis of plants, animals and microbes. In: Pond EG, Bazer FW, Rollin BE (eds) Animal welfare in animal agriculture: husbandry, stewardship and sustainability in animal production. CRC Press, New York, pp 185–120

Zivcak M, Brestic M, Kunderlikova K, Olsovska K, Allakhverdiev SI (2015) Effect of photosystem I inactivation on chlorophyll *a* fluorescence induction in wheat leaves: does activity of photosystem I play any role in OJIP rise? J Photochem Photobiol B Biol 152:318–324

Zuccaro A, Lahrmann U, Langen G (2014) Broad compatibility in fungal root symbioses. Curr Opin Plant Biol 20:135–145

Chapter 5
Targeted Gene Disruption Tools for Fungal Genomics

Ramesh N. Pudake, Maya Kumari, Binod Bihari Sahu, and Eram Sultan

Abstract Advances in genetic sequencing of fungal genomes have made vast genetic resources available for improvement of these economically important organisms. For this various functional genomics tools like gene cloning, transformation, and mutations have been developed and are being used. Gene function can be studied both by forward genetics and reverse genetics. Mutant generation and characterization have been an exceptional tools for studying the role of specific gene in the development of fungi. In this chapter, various molecular techniques which can be utilized for controlling the expression of specific genes in fungi are described. For this we need to insert external DNA molecules in fungal cells through genetic transformation followed by the analysis of gene function. This chapter also focuses on the novel strategies for gene-editing techniques such as ZFNs and TALENs which can also be used to elucidate the functions of genes. These molecular tools for targeting gene expression have greatly enhanced the knowledge of fungal genes functions.

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5.1 Introduction

Fungi are the eukaryotic organism which has over 1.5 million members. These organisms are present in almost all places of the Earth and affect almost all other forms of life. They can be a boon or bane to human being. They are majorly involved in decomposition of organic matter, which is very critical in the maintenance of environmental cycling of organic matter (James et al. 2005). The members of fungal kingdom exist from microscopic nature to the large mushrooms and possess a huge biodiversity. Due to their decomposing ability, the fungus plays an important role in ecological cycling. Along with this they also possess an economical importance and contribute to the world economy by producing various by-products ranging from food to medicines. In contrast to these beneficial roles, some of the members of the fungal kingdom are major disease-causing organisms in both plants and animals (Tsang 2014). Losses due to fungal diseases are a major concern as it is directly related to worldwide food security (Fisher et al. 2012). Due to the faster evolution of plants, their association with fungi is wider as compared to the animals. This is proved by the fact that as compared to the number of animal disease-causing fungi, the number of plant disease-causing fungi is manyfold (Horbach et al. 2011).

During the evolution period, the plant pathogenic fungi have advanced the methods of invading the plant immunity. Based on the method of invasion, they are categorized as necrotrophs or biotrophs. While necrotrophs infect and kill the host tissues, the biotrophs possess a sustained pathogenesis strategy. Necrotrophs generally produce toxin to kill the host cells; biotrophs lack the toxins but continually derive nutrients from the host cells by haustoria. There is another intermediate way of infection where at the initial stages of infection the pathogen possesses biotrophic phase and necrotrophic nature is displayed at the later stages. These fungi are called hemibiotrophs (Horbach et al. 2011).

These host-pathogen interactions and its impacts can only be understood by studies at cytological and genomic levels. Since the invention of the microscope, the studies are being conducted at cellular level. But to study the fungal processes at gene level, the information at genome is required. First genome of baker's yeast (*Saccharomyces cerevisiae*) was sequenced in 1996 (Dujon 1996). This acts as the beginning, and to get valuable data from important fungi about their role in basic research, health, agriculture, and industry through genomics, a project to sequence 1000 fungi was initiated through Fungal Genome Initiative of the US Department of Energy's Joint Genome Institute (JGI) (Grigoriev et al. 2011).

Currently, about 100 important fungi have been sequenced and many more are being sequenced. Analysis of these sequence information can provide an insight about genes that are involved in host-pathogen interaction or involved in the production of economically important substances. Presently, functional and comparative genomic studies in fungi are at initial stages but have enormous potential in

understanding the bioprocess involved in the life cycle of fungi. To achieve this, there is an immediate need of advanced genomic tools and infrastructure for efficient utilization of the vast wealth of available genome sequence information. This will provide a platform for systemic studies of fungal kingdom. Gene function can be deciphered in two ways—forward genetics and reverse genetics (Himelblau et al. 2009). Mutant generation and characterization have been an exceptional tool for studying the role of specific gene in the development of fungi. Generally, mutants are generated by chemical or physical mutagens, and those are randomly interspersed within the genome of organism. Mutagenesis of this kind also suffers several other drawbacks like lower frequency, lethal effects, high operative risks, etc. More importantly, no clue could be obtained as to where in the genome and in which gene the mutation has occurred or whether it is due to a single base change, an insertion, or a deletion.

As molecular biologists have developed various techniques to clone the gene and sequence it, it has became routine now to isolate a single gene. So classical random mutation technique also needs refinement. The gene-specific mutations could be induced by various new technologies. The process to achieve silencing of targeted genes by classical modern PCR-mediated process can be called as *site-directed mutagenesis* which is now a popular tool for gene manipulation in all kinds of organisms (Smith 1986). The availability of complete or draft genome sequences of many fungi makes them more suitable for research on topics such as cell biology, genetics, signal transduction, and differentiation (Borkovich et al. 2004) by mutagenesis. This chapter focusses on the methodologies of site-directed mutagenesis and its application in fungal genomics.

5.2 Methodologies for Targeting Specific Gene in Fungi

In majority of cases, the importance of a gene cannot be determined by simply recognizing amino acid motifs in the protein and by examining the close relatives. In order to establish the functional relationship between gene and phenotypes through reverse genetics approach, generation of null mutant is required. The gene of interest for studying its function is subjected to in vitro site-specific mutagenesis, thereby generating a loss- or gain-of-function mutant to detect a phenotype that may provide some clue about function. Not surprisingly, during the recent years that gene targeting techniques have been available, many genes have been knocked out in fungi. The methods employed in fungi for disrupting a gene's open reading frame and to block its expression are homologous integration, ectopic integration, and RNA silencing.

5.2.1 Homologous Integration

Homologous integration of vector DNA for targeted gene replacement takes advantage of an organism's own DNA repair machinery. The error-free DNA replication during cell multiplication is indigenously achieved by homologous recombination (HR). This is common in all forms of life, which ensures high-fidelity, template-dependent repair or tolerance of complex DNA damages including DNA gaps, DNA double-stranded breaks (DSBs), and DNA interstrand cross-links (ICLs). Along with its involvement in preserving the genome during cell division, HR plays a prominent role in faithfully duplicating the genome by providing critical support for DNA replication and telomere maintenance (Li and Heyer 2008). HR in eukaryotes is carried by a conserved member of the RAD51 protein family—Rad51 recombinase—that is homologous to bacterial RecA. The process requires a nick or break in dsDNA that allows invasion of a 3′ overhang of the broken DNA to the non-broken homologous DNA molecule. Lastly, strand elongation occurs in order to restore damage following either of the two main pathways—the DBSR (double-strand break repair) pathway or the SDSA (synthesis-dependent strand annealing) pathway (Heyer et al. 2010; San Filippo et al. 2008). Repair of a DSB proceeds either through the DSBR mechanism, which results in crossovers or is more common in meiosis than in mitosis, or through the SDSA mechanism (right), which results in noncrossovers.

Gene targeting by homologous integration consequently leads to gene disruption or gene replacement (knockout, knock-in or knockdown). Gene disruption involves exchange of genetic segment between genomic and exogenous DNA molecules via crossover events (Reh and Vasquez 2001). Generally, the exogenous DNA molecules contain a selectable marker—a drug or antibiotic-resistant gene—flanked on both sides with the genomic segments of target gene which will act as template for homologous recombination. The size of these flanked homologous sequence, the transformation method, and the chromosomal location of gene are the major factors which are responsible for the efficiency of gene knockout by replacement vector (Weld et al. 2006). When the optimization of these factors is acheived, homologous recombination results in an excision and exchange phenomena with the help of indigenous recombinases (Fig 5.1). Conditional gene knockout via homologous recombination was achieved in fungi by many researchers (Table 5.1).

5.2.2 Genetic Engineering by RNA Interference

In addition to DNA-based approaches for site-directed mutagenesis, RNA interference is a great tool for reducing the gene expression in eukaryotes. RNA interference (RNAi) is a natural defensive phenomenon against mobile genetic entities

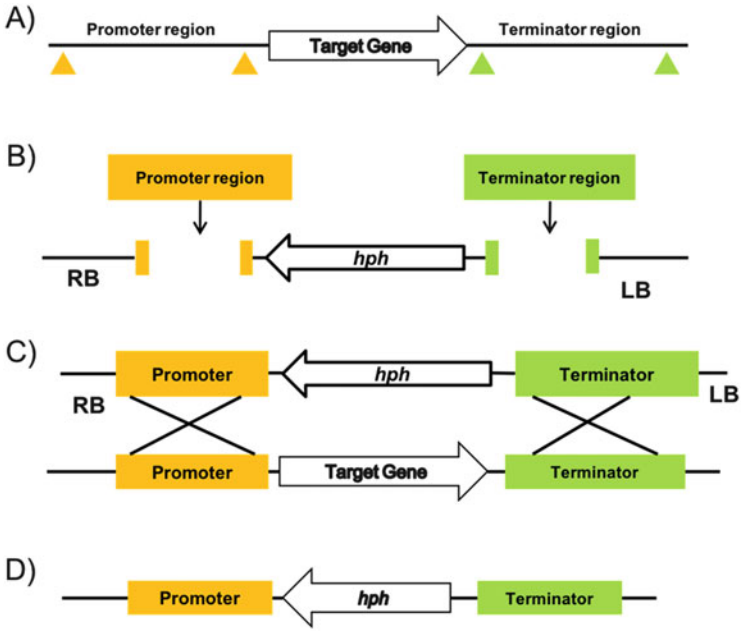

Fig. 5.1 (**a**) A schematic of construction of replacement vector for gene silencing through homologous recombination. The two homologous segments are cloned in multiple cloning sites flanked with selective marker. When linearized for gene targeting, the vector backbone will then replace the target gene with selectable marker by homologous recombination. (**b–d**) Gene replacement by homologous recombination with selection marker. Two targeting arms (homologous sequence) are depicted in *yellow* and *green colors*. Two homologous recombination events (depicted through the *crosses*) occur *via* the targeting arms to introduce nonhomologous sequence (e.g., *hyg*) into the designated gene. Insertion of the hygromycin gene is selected by growing transformants on media with hygromycin. These mutants need to confirmed by various tools like Southern blotting or gene-specific PCR because if the targeting construct is randomly integrated anywhere in the genome, the gene of interest would be intact

such as viruses and transposons in a spatiotemporal manner, capable of producing aberrant RNA or dsRNA (Sioud 2015). The mechanism first studied in the nematode, *Caenorhabditis elegans*, is also differently referred to as posttranscriptional gene silencing. It uses a small noncoding RNAs of 20–30 nucleotides (which fold back forming hairpin) produced from double-stranded RNA to regulate the post-transcriptional gene expression (Chang et al. 2012). In this method these small RNAs regulate the similar genes by digesting the mRNA. On exposure to dsRNA, cells activate a series of events that eventually leads to degradation of the corresponding mRNA and gene silencing. RNAi-like silencing in organisms can be introduced via siRNA, miRNA, PIWI-interacting RNAs, etc. Based on their precursor structures, origin pathways, and mode of action, small RNAs are majorly divided in four categories—small interfering RNA (siRNAs), microRNA (miRNAs), PIWI-interacting RNA (piRNAs), and primal small RNAs (priRNAs) (Ghildiyal and Zamore 2009).

Table 5.1 Details of targeted gene disruption studies in different fungi

Fungus species	Gene studied	Method utilized	Transformation method	Phenotype/character	References
Acremonium chrysogenum	DsRed Reporter Gene	RNA silencing	DNA-mediated transformation	Efficient silencing of *DsRed* expression	Janus et al. (2007)
Aspergillus flavus, A. parasiticus, Fusarium graminearum	AflR (*Aspergillus sp.*), Tri6 (*Fusarium*)	RNA silencing via inverted repeat sequences	Protoplast transformation	IRT-induced loss of toxin (aflatoxin, versicolorin A) production is stable during infection of host plants, and, in the case of *F. graminearum*, a decrease in virulence results from deoxynivalenol suppression	McDonald et al. (2005)
Aspergillus fumigatus	*aoxAf*—mitochondrial alternative oxidase gene	RNA interference (RNAi)	*Agrobacterium tumefaciens*-mediated transformation	Enhances reactive oxygen species production and killing of the fungus by macrophages	Magnani et al. (2008)
Aspergillus nidulans	nkuA (Human KU70 homolog)	Homologous recombination	Protoplast Transformation	Greatly reduces the frequency of nonhomologous integration of transforming DNA fragments, leading to dramatically improved gene targeting	Nayak et al. (2006)
Aspergillus nidulans	LaeA	–	Protoplast transformation	This nuclear protein transcriptionally activates several secondary metabolite gene clusters	Bok et al. (2006)
Aspergillus oryzae	AoAtg1	Homologous recombination	Protoplast transformation	Mutant lacked aerial hyphae and conidiation, and autophagy was likely completely inhibited	Yanagisawa et al. (2013)
Bipolaris oryzae	PKS1—a polyketide synthase gene	RNA interference (RNAi)	–	Resulted in albino phenotypes	Moriwaki et al. (2007)
Botrytis cinerea	BcnoxD	Homologous recombination	Protoplast transformation	Deletion mutants produced less conidia as compared to wild type	Ulrike et al. (2015)
Candida albicans	RAD52	Homologous recombination	Protoplast transformation	HR plays an essential role in the repair of DNA lesions	Ciudad et al. (2004)

Colletotrichum graminicola	β-1,3-glucan synthase gene *GLS1*	RNA interference (RNAi)	Protoplast transformation	Reduced infection of maize host	Oliveira-Garcia and Deising (2013)
Colletotrichum higginsianum	ChKU70	Homologous recombination	*Agrobacterium tumefaciens*-mediated transformation	Increase in the frequency of integration of introduced exogenous DNA fragments	Ushimaru et al. (2010)
Fusarium virguliforme	*FvTox1*	Homologous recombination	*Agrobacterium tumefaciens*-mediated transformation	*FvTox1* toxin is involved in foliar sudden death syndrome development in soybean	Pudake et al. (2013)
Grosmannia clavigera	The polyketide synthase (PKS) and the scytalone dehydratase (SD)	Homologous recombination	*Agrobacterium tumefaciens*-mediated transformation	Affects melanin biosynthesis pathway	Wang et al. (2010)
Magnaporthe grisea	mus52/KU80	Homologous recombination	Protoplast transformation	Targeted gene replacement frequency was increased	Villalba et al. (2008)
Magnaporthe grisea	ACE1—a polyketide synthase (PKS)	Single homologous recombination	*Agrobacterium tumefaciens*-mediated transformation	Required for avirulence	Collemare et al. (2008)
Melampsora lini	AvrL567—a rust effector protein	RNA interference (RNAi)	*Agrobacterium*-mediated genetic transformation	*AvrL567* gene is responsible for flax rust virulence phenotypes	Lawrence et al. (2010)
Metarhizium anisopliae	*trp1* locus	Homologous recombination	*Agrobacterium tumefaciens*-mediated transformation	Unable to grow in tryptophan-lacking media	Staats et al. (2007)
Moniliophthora perniciosa	GFP and a peroxiredoxin	RNA interference (RNAi)	–	Reduction of GFP and peroxidase mRNA levels	dos Santos et al. (2009)
Penicillium decumbens	pku70	Double Homologous recombination	Protoplast transformation	Efficiency of gene targeting increased	Li et al. (2010)

(continued)

Table 5.1 (continued)

Fungus species	Gene studied	Method utilized	Transformation method	Phenotype/character	References
Phanerochaete chrysosporium	Manganese-containing superoxide dismutase gene (MnSOD1)	RNA interference (RNAi)	Electroporation	Decrease the enzymatic activity	Matityahu et al. (2008)
Hypocrea jecorina/ Trichoderma reesei	G-alpha protein GNA3	RNA interference (RNAi)	Protoplast transformation	Regulates cellulase gene expression in the presence of light	Schmoll et al. (2009)
Trichoderma atroviride	Tga3, a Novel G Protein α Subunit	Homologous recombination	*Agrobacterium*-mediated transformation	Role in vegetative growth and can alter mycoparasitism-related characteristics, such as infection structure formation and chitinase gene expression	Zeilinger et al. (2005)
Hypocrea jecorina (*Trichoderma reesei*)	A PAS/LOV Domain Protein, env1	Homologous recombination	Protoplast transformation	Modulates cellulase gene transcription in response to light	Schmoll et al. (2005)
Hypocrea jecorina (*synonym: Trichoderma reesei*)	Human KU70 orthologue	Homologous recombination	Protoplast transformation	Efficiency of gene targeting by homologous recombination was increased	Guangtao et al. (2009)
Trichoderma virens	gliP	Homologous recombination	Protoplast transformation	Deletion of gliP abolishes gliotoxin production Δ*gliP* strains demonstrated considerable sensitivity to the oxidative stress	Vargas et al. (2014)
Trichoderma virens	Hydrophobin-like elicitor Sm1	Homologous transformation	Protoplast transformation	Required for induced systemic resistance in maize	Djonović et al. (2007)
Trichoderma virens	Carbamoyl phosphate synthase, arg2	Homologous transformation	Protoplast transformation	–	Baek and Kenerley (1998)
Trichoderma virens	Adenylate-cyclase-encoding gene, tac1	Homologous transformation	Protoplast transformation	Lowered the intracellular cAMP	Mukherjee et al. (2007)
Ustilago maydis	Pep1	Homologous recombination	Protoplast transformation	Mutants fail to establish a biotrophic interaction immediately after entry into the host plant	Doehlemann et al. (2009)
Trichoderma virens	Mitogen-activated protein kinase encoding gene, tvk1	Homologous recombination	Protoplast transformation	A negative modulator during host sensing and sporulation	Mendoza-Mendoza et al. (2003)

Trichoderma atroviride	G protein alpha subunit Tga1	Homologous recombination	*Agrobacterium*-mediated transformation	Regulate the biosynthesis of different antifungal substances	Reithner et al. (2005)
Trichoderma atroviride	*tmk1* gene—a mitogen-activated protein kinase	Homologous recombination	*Agrobacterium*-mediated transformation	Deletion caused reduced mycoparasitic activity	Reithner et al. (2007)
Trichoderma virens	G-protein Alpha subunits, *TgaA and TgaB*	Homologous recombination	Protoplast transformation	Both TgaA and TgaB mutants lysed the mycelia of *R. solani*, but TgaA mutants had reduced ability to colonize *S. rolfsii* colonies	Mukherjee et al. (2004)
Trichoderma atroviride	G-protein alpha-subunit gene tga1	–	Biolistic transformation	Mycoparasitic signaling was reduced	Rocha-Ramirez et al. (2002)
Trichoderma virens	VELVET protein Vel1	Homologous recombination	Protoplast transformation	Defective in secondary metabolism (antibiosis), mycoparasitism, and bio-control efficacy	Mukherjee and Kenerley (2010)
Trichoderma reesei	Cellobiohydrolase II (CEL6A)	RNAi	–	Stable decrease in levels of cel6a mRNA and CEL6A protein	Brody and Maiyuran (2009)
Aspergillus niger	Amyloglucosidase (glaA) gene	RNAi	–	Complete suppression of the amyloglucosidase (glaA) gene	Brody and Maiyuran (2009)
Trichoderma reesei	Cla4, Ras2, and RhoA and eGFP	RNAi	–	With dual promoter construct the transformants were selected rapidly by using eGFP as a reporter	He et al. (2015)

5.2.2.1 Small Interfering RNA

Involvements of RNAIII family members cleave the long dsRNA into 19–23 nts fragments having 5′ phosphorylated end including unphosphorylated 3′ ends which may be of two kinds, endogenous and exogenous (as defense pathway)—siRNAs as per their origin. These siRNAs thus formed constitute the siRNA duplex that consists of passenger strand as well as guiding strand. Release of the passenger strand occurs when only the RNA interference specificity complex (RISC) uses the guide strand that is used to find the target mRNA for endolytic cleavage. They create dsRNA precursor for dicer. The use of RNAi-based strategies for plant breeding in crop improvement is in practice. Also, this particular phenomenon downregulates the invading bacterial, fungal pathogens inside the host. Virus-induced gene silencing (VIGS) is one of the arenas of research where in the antiviral defense mechanism is studied.

5.2.2.2 MicroRNA

In order to unravel the possibility to analyze the involvement of a particular set of genes for phenotype under a certain condition, one does not need to screen a whole lot of mutants; rather, microRNA (miRNA) based technology to control the gene expression of a set of genes can be used. The designing of the concerned vectors and putting them in the host of interest can easily target PAMPs or pathogen-derived effector molecules. Further, tissue-specific targeting and downregulating the expression of a subset of genes can be achieved by this process artificially which is achieved by the degradation of the target mRNA at 10–12 bps complementary to the miRNA designed and transformed. It has already been achieved in tomato, rice, and tobacco by various groups of institutions.

5.2.2.3 PIWI-Interacting RNA

These are a group of endogenous small RNAs consisting of 24–31 nts length and after associating with PIWI proteins, and Tudor superfamily members (in *Drosophila*, many more proteins are also involved, Vasa (Vas), Maelstrom (Mael), Armi, Zuc, Squash (Squ), and Shu) are called piRNA-induced complexes and are further methylated for their stability in vivo (Siomi et al. 2010) in a ping-pong cycle (Brennecke et al. 2007). This is also similar to the amplification of secondary siRNA in eukaryotes. piRNAs repress the transposon in order to maintain the integrity of the genome of an organism as guardian of the genome at transcriptional or posttranscriptional level. They are derived from wide intergenic regions of repetitive elements in clusters which otherwise was called as junk DNA part in earlier days (Lau et al. 2009; Saito and Siomi 2010). However, down the line of generation also, it is inherited for many (multi)generations epigenetically, which

is amid the involvement of the concerned piRNAs in the regulation of the transposon-based regulation of the concerned cellular or physiological process. Their sequences are having antisense orientation as that of transposons and transcripts to be targeted by the physiological affect.

5.2.2.4 Primal Small RNAs

Mediated by exoribonuclease activity for 3′ end formation and independent of Dicer, priRNAs resemble piRNAs. They are mostly derived from 3′UTR region and degradation products of various transcripts of the respective genes in a Triman-dependent manner, which might be functioning for surveillance of mRNAs by interrupting the genome from being incorporated with foreign genomic elements (Halic and Moazed 2010; Marasovic 2015). Understanding the biogenesis and their involvement for regulation of the genome at transcriptional level is under study.

5.2.3 Zinc Finger Nucleases and Engineered Meganucleases

The challenges in genome editing *via* targeted gene replacement are low specificity, generation of unexpected mutants, and low efficiency of mutagenesis. So recent techniques like zinc finger nucleases (ZFNs) and engineered meganucleases are of potential for specific targeting and modification of defined DNA sequences *in vivo* (Baker 2012). In this method, the sequence-nonspecific DNA cleaving enzymes are fused to sequence-specific recognition DNA-binding protein. Zinc finger proteins are used as the hand of this engineered scissor, because of its capability of recognition of specific nucleotides (Carroll 2011). In yeast the transcription activator-like effectors (TALEs) were fused to Fok-I nucleases to create the TALE nucleases (TALENs) for targeting DNA double-strand breaks (Christian et al. 2010). Theoretically, any sequence of interest can be used to design customized TAL code which can accurately recognize it and cleave the side gene sites (Cermak et al. 2011). Recently a rapid and efficient-Fast Ligation-based Automatable Solid-phase High-throughput (FLASH) system was developed for gene editing in humans (Reyon et al. 2013). These studies act as a base of many studies in plants, yeast, and many other organisms to induce heritable mutants .The successful application of TALEN for gene editing in yeast have open up the possibility of using it in the fungal kingdom, but still at present there are no reports of usage. The mechanism used by organism to repair the TALEN-triggered break in genomic DNA is either nonhomologous end joining (NHEJ) or HR pathway (Reyon et al. 2013). If the template for homologous repair is available after double-strand breaks (DSBs) had occurred at the site of interest, the frequency of HR is improved (Bedell

et al. 2012). So outcomes like site-directed mutation, terminal tagging, and gene integration can be achieved. Recently a lot of modifications in gene-editing techniques are going on, and surely one day it will play a very important role in fungal genomics.

5.3 Vector Design for Various Methods of Targeted Gene Disruption

5.3.1 Homologous Recombination

For occurring of a recombination event in a fungal cell, around 2 kb of sequence homology is required. However, less than 1 kb of homology is not typically recommended for targeting constructs (Frandsen 2011; Krappmann et al. 2006). In addition, linear DNA was found to be preferred for transferring the recombinant constructs. The frequency of homologous recombination is very less in a nonhomologous end joining (NHEJ) genetic background as compared to random insertion. Chemical competency transfers of linear DNA in fungal protoplast and *Agrobacterium*-mediated transformation are preferred ways to deliver the replacement vectors into fungal cells. The vector employed for this event is called a replacement vector, in which open reading frame of genomic clone is disrupted by the placement of an intervening drug selection marker. And two events of homologous recombination inserting the selection marker at targeted genomic locus (Fig. 5.1) and positive transformants are selected. Hygromycin is the most common drug used for positive selection (Table 5.1).

The frequency of gene knockout by double HR has been significantly increased by the generation of mutants with defects in the NHEJ system (*ku70*, *ku80*, or *lig4* mutants) (Krappmann et al. 2006). The deficient NHEJ system in fungi forces the T-DNA of *Agrobacterium* to integrate into genome through the HR system (van Attikum et al. 2001), which ultimately results in increased events of gene knockout. But in many cases of fungal transformation, there is no requirement of mutation in NHEJ system for achieving site-directed gene replacement through homologous recombination (Frandsen 2011; Pudake et al. 2013). The gene knockout frequency in fungi can also be increased by another approach, where the gene targeting cassette is flanked with a negative selection marker. This marker is lost if the vector DNA integrates in fungal genome via the HR pathway but can be preserved if the DNA integrates *via* the NHEJ pathway. This strategy removes the possibility of transformants' selection which carries ectopic copies of the introduced DNA and increases desired gene replacement pool (Gardiner and Howlett 2004). Though the use of both positive and negative selection reduces the required screening work, the gain can be considered marginal due to the high rate of false positives. In addition to

the use of NHEJ-deficient strains and negative selection marker systems, the split-marker strategy is a promising technique for increasing the gene targeting efficiency in ATMT experiments (Wang et al. 2010).

5.3.2 RNAi-Mediated Gene Silencing in Fungi

RNAi-mediated gene silencing as a natural phenomenon is triggered efficiently by the dsRNAs. Likewise, targeted gene silencing can be achieved by introduction of various small RNAs like siRNAs (Fig. 5.2), miRNAs (Fig. 5.3), PIWI-interacting RNAs (piRNAs), and primal small RNAs (priRNAs) into a cell that can associate with several proteins such as Dicer/Argonaute and PIWI/aubergine to follow the natural RNAi pathway. Small RNAs can be introduced into a cell by liposome-based transfection or electroporation of mimics, synthetic constructs, or a vector carrying an insert thereof. Small RNA inserts are synthesized such that they would result in a self-complementary, hairpin-like precursor RNA transcript. An intronic sequence of target mRNA and reverse compliment thereof, flanked by restriction sites, is selected by processing and checking through RNAi Designer software available, and cloned in vector. Further, cross-checking of the inserted fragment which can form hairpin-like structures is framed in repetition by sequencing and digestion tests. Once conformation of the cloning of the cassette is confirmed, it is put downstream of a constitutive promoter in binary vector that would be helpful for recombining into the genome of the species of concern. Moreover, the insert comprises nucleotide sequences that would correspond to sense and antisense strands of the stem of hairpin, separated by a spacer sequence that would form the loop of the hairpin (Fig. 5.2).

5.4 Transformation Technologies for Site-Directed Mutagenesis Strategy

The success of any attempt to achieve the gene knockout or silencing is depending on the method of gene transfer by transformation (genetic transformation). It is an indispensable technique for such studies and has provided powerful tools for evaluating fungal gene functions. The transformation fungus can be achieved by using two major methods, like protoplast transformation and *Agrobacterium tumefaciens*-mediated transformation (ATMT).

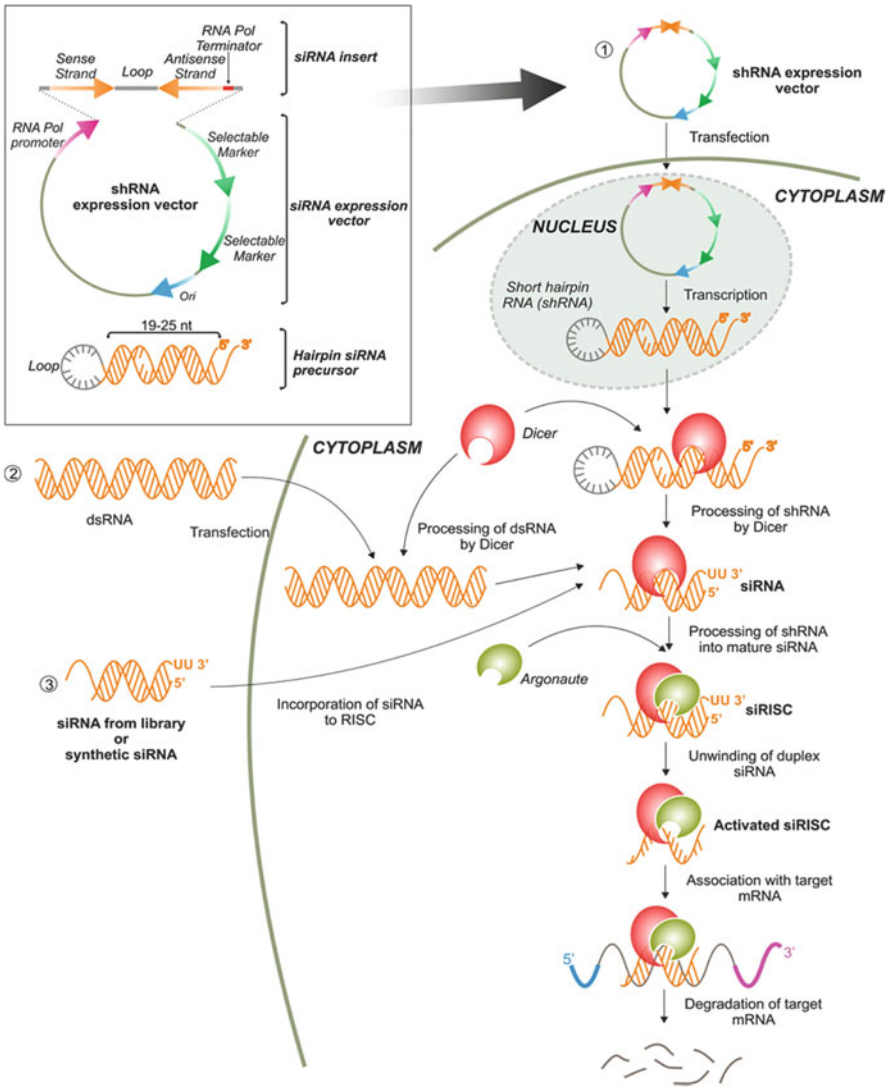

Fig. 5.2 siRNA-mediated induced gene silencing. Three methods for siRNA-mediated induced silencing of genes. (1) Vector-based siRNA induced silencing. A vector construct (inset) with a short hairpin RNA (shRNA) insert downstream of a constitutive promoter is introduced into cell *via* liposome-mediated transfection. The shRNA transcript comprises a self-complimentary 19–25 nt stem of sense and antisense strands and a 6 nt loop. The shRNA transcript is processed by Dicer into siRNA. Dicer-siRNA complex is further accompanied by Argonaute which unwinds duplex miRNA, forming an activated siRNA-induced silencing complex (siRISC). siRISC then interacts with target mRNA leading to gene silencing by target mRNA degradation by endolytic nucleases. (2) Silencing by dsRNAs. dsRNAs, usually longer than 30 nt, undergo processing by Dicer and associate with Argonaute to form siRISC which follows a similar silencing pathway. (3) Silencing by synthetic siRNA. siRNAs can be synthesized and transfected in its mature form, which incorporate in RISC with Dicer and Argonaute and silence the targeted gene by mRNA degradation

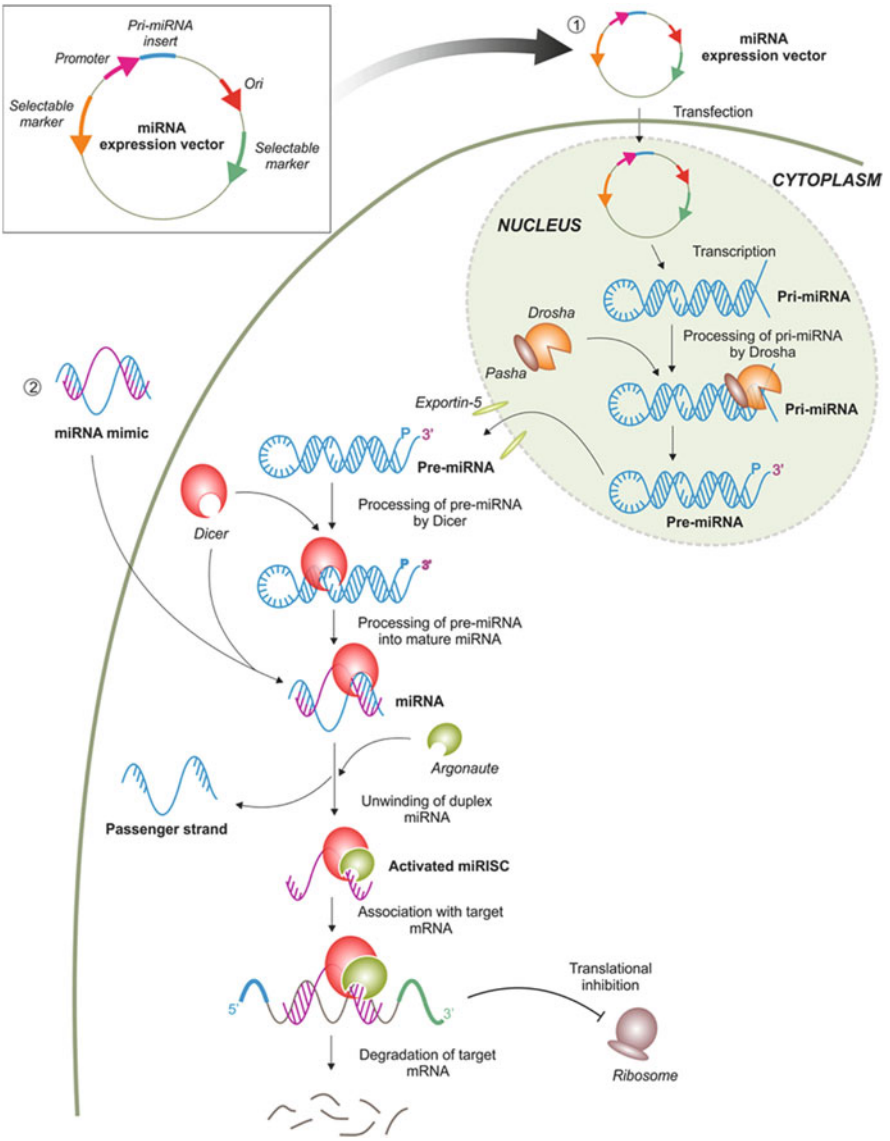

Fig. 5.3 miRNA-mediated induced gene silencing. Two methods for miRNA-mediated induced silencing of genes. (1) Vector-based miRNA induced silencing. A vector construct (inset) with a pri-miRNA insert downstream of a constitutive promoter is introduced into cell *via* liposome-mediated transfection. The pri-miRNA transcript is processed by endogenous Drosha-like nuclease into miRNA precursor (pre-miRNA). The modified 3′ end of pre-miRNA aids in transportation to cytoplasm *via* Exportin-5 where it is further processed by Dicer into miRNA. Dicer-miRNA complex is further accompanied by Argonaute which unwinds duplex miRNA, forming an activated miRNA-induced silencing complex (miRISC). miRISC then interacts with target mRNA leading to gene silencing either by translation inhibition or by target mRNA degradation following decapping and deadenylation. (2) Silencing by miRNA mimics. Mimics are synthetic mature miRNAs that can be directly transfected into the cell. In the cytoplasm, miRNA mimics interact directly with Dicer and Argonaute forming miRISC which initiates gene silencing by

5.4.1 Physical Methods of Fungal Transformation

5.4.1.1 Electroporation

Electroporation is the most common and popular physical technique for bacterial transformation (Yoshida and Sato 2009). But it is also used in the transformation of yeast and fungi. It uses strong electrical field to cells or tissues to transfer the DNA inside the cells. It is a very simple, quick, and efficient transformation method as compared to other methods, but still it needs appropriate adjustment in parameters for each fungal species. Sometimes special treatment of fungal cells or tissues is needed to achieve better results.

When pulsed or alternate electric fields are applied to living cells, it results in the formation of pores due to polarity alteration on plasma membrane. This allows the external DNA molecules to enter inside the cell. Generally, voltage between 0.5 and 2 V is applied, and pore formation in membrane is considered to be formed due to the transitory force of electrode formation produced by the electrostatic interaction of the dipoles generated on the cells. The exogenous DNA is captured in cells through these pores. The time and strength of the electrical pulse are the critical factors for successful transformation. The success rate of electroporation is also dependent on concentrations of DNA and tolerance of cells to membrane permeation, and the heterogeneity of the cell population may also affect the electrotransfection efficiency (Rivera et al. 2014). The major advantage of electroporation lies in its applicability in all kinds of fungi and also in actively growing fungal cells or frozen samples. Efficient protocols can be easily optimized with little efforts. However, the major drawback is the low transformation efficiency due to electrophysiological characteristics of the fungus.

The electroporator used for genetic transformation is a container with a slot containing two parallel aluminum electrodes which are in contact with an aqueous electrolyte, containing intact cells/protoplast in suspension and the DNA desired to be incorporated into the cell. Commonly electrical pulses lasting from microseconds to milliseconds with a voltage between 1 and 2 kV are used for fungal transformation. But in some fungal species, increasing the field strength has been reported to enhance transformation efficiency also. The transformation efficiencies of fungi range from 10^3 to 10^6 transformants/μg of DNA (Rivera et al. 2014).

5.4.1.2 Biolistic Transformation

Biolistic transformation is popularly known as “particle bombardment” or the “gene gun technique” of genetic transformation. This is one of the popular

Fig. 5.3 (continued) decapping, deadenylation, and ultimately degradation of target mRNA or by obstructing the assembly of translational machinery

transformation methods because of its universal usage (Rakoczy-Trojanowska 2002; Sanford 1988). It is very quick and less costly as compared to other methods of transformation. The possibility of introducing multiple genes in one step can only be easily done in this method (Rivera et al. 2014). In this system of genetic transformation, some high-density carriers like gold and tungsten are coated with DNA. The size of these carrier particles is kept smaller than fungal cells, and so when they enter cells, the DNA also gets an entry. This technique was first used for transformation in cereals in 1987 and since then is being regularly used in plant, yeast, and algal species (Wang et al. 2013). The transformation efficiency of this method depends on various parameters, like the number of cells bombarded, the quantity of DNA-coated particles, the quantity of DNA on each particle, and the particles' kinetic energy. The regeneration efficiency of cells is also positively correlated to transformation efficiency. According to the reports, the transformation efficiencies in fungi by biolistics method range from 10^4 to 10^5 transformants/μg of DNA.

Biolistics is being used by many researchers for site-directed mutagenesis. This is a very useful method for genetic transformation of fungi as it does not need any enzymatic removal of cell walls. The viability of cells is not reduced which results in better regeneration. Another advantage of this method is the selection of tissues for transformation. The tissues like conidia or mycelium can be transformed by biolistics. However, the need for sophisticated device, particle gun, and the possibility of high copy number of transgene are major limitations. Additionally, low frequencies of transformation are commonly obtained.

The equipment used for transformation consists of two chambers—one with a high pressure and second one with a low pressure. These chambers are separated by a diaphragm in the middle that accelerates DNA-covered microparticles of gold, tungsten, or platinum (Montanari et al. 2014). As the microparticles hit the cells, the DNA molecules covering it are released and may be integrated into chromosomal DNA of organism by adsorption mechanism. For fungal transformation the helium gas pressure which ranges from 500 to 2000 psi is used, and the micro-projectiles are accelerated to speeds of 400 m/s or higher in a partial vacuum of about approximately 30 mm Hg. There is no need of vector-specific sequences; also this method is not dependent on the electro-physical properties of cells. The transformation parameters need to be modified specifically according to tissues or species used. The possibility of introducing transgene at multilocus results in low stability of transgene is a major disadvantage of this method, still the biolistics has been used to transform many filamentous fungi (Table 5.1).

5.4.2 Biological Method of Transformation

The fungal transformation for site-directed mutations by biological method is based on *Agrobacterium tumefaciens*-mediated transformation (ATMT). *Agrobacterium* is a Gram-negative plant pathogenic bacterium, which has the ability to create

crown gall tumors in plants. *A. tumefaciens* can transfer the T-DNA region (200 kb) from the tumor-inducing plasmid (Ti plasmid) to the host plant nuclear genome. The ATMT has long been used for genetic manipulation of a wide variety of higher plants. In 1998, a method for the transformation of filamentous fungi was introduced that was derived from the successful plant transformation system based on the soil bacterium *A. tumefaciens* (de Groot et al. 1998). Recently, ATMT was extended to molecular genetic studies of filamentous fungi (Sugui et al. 2005), most of which showed markedly higher transformation frequencies than traditional methods, such as the protoplast/PEG-mediated method. In addition, the majority of the transformants obtained by ATMT showed integration of T-DNA at single-locus T-DNA into the genome, which is advantageous for specific manipulation of endogenous genes, e.g., targeted gene disruption and insertional mutagenesis. ATMT is regarded as an efficient reverse genetics experimental tool for functional genomics research in many filamentous fungi. Its application has been widely used for studies involving gene knockout, overexpression, and complementation and generating random integrations. At present, over 180 general binary vectors yielding different degrees of adaptability or infectivity in the host have been successful in fungal transformation (Frandsen 2011).

5.5 Conclusions and Prospects

Fungi—a class of lower eukaryotes—possess unique physiological features that have been involved in beneficial and pathogenic reactions and consequently have attracted the attentions of researchers. In the past, scientists all over the world have made efforts for understanding the functional relationship of fungal genes by using various molecular tools. The studies were planned for studying pathogenicity, development, reproduction regulation, cell autophagy, and metabolic pathways and seeking new interesting genes. Molecular tools like transformation by *Agrobacterium*, electroporation, and gene gun were developed for plants/animals and are now being commonly used for fungi. Methods for the construction of random mutant libraries including ATMT were created and being used for analyzing the phenotypes resulting from mutation in genes. Subsequently, RNA interference technique which takes an advantage of biological process of regulating gene expression was widely used. Novel gene editing techniques such as ZFNs and TALENs were also used to elucidate the functions of genes. These molecular tools for targeting gene expression have greatly enhanced the knowledge of fungal genes' functions. However, every tool discussed in this chapter has advantages and limitations. One may be more suitable in one organism, but may be less in another. Therefore, it is very important to choose a suitable tool and strategy for gene silencing in different fungi. If anybody ignores the technological obstacles, it may result in loss of time and labor. So systematic planning is necessary since initial stages, to take advantage of current developments. The application of novel techniques, such as comparative genomics, transcriptomics, proteomics, and

metabolomics, will provide abundant information to allow us to better understand the molecular mechanisms governing cellular processes and pathogenicity. Those advances will open new doors to better engineer industrial strains and will certainly contribute to the drug design against pathogenic fungi. In the future, the application of functional genomics in filamentous fungi will be more broadly adopted. Furthermore, novel techniques with increased automation, extreme high efficiency, decreased costs, and greater information content will likely be developed.

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References

Baek JM, Kenerley CM (1998) The arg2 gene of Trichoderma virens: cloning and Development of a homologous transformation system. Fungal Genet Biol 23:34–44

Baker M (2012) Gene-editing nucleases. Nat Methods 9:23–26

Bedell VM, Wang Y, Campbell JM, Poshusta TL, Starker CG, Krug Ii RG, Tan W, Penheiter SG, Ma AC, Leung AYH et al (2012) In vivo genome editing using a high-efficiency TALEN system. Nature 491:114–118

Bok JW, Noordermeer D, Kale SP, Keller NP (2006) Secondary metabolic gene cluster silencing in Aspergillus nidulans. Mol Microbiol 61:1636–1645

Borkovich KA, Alex LA, Yarden O, Freitag M, Turner GE, Read ND, Seiler S, Bell-Pedersen D, Paietta J, Plesofsky N et al (2004) Lessons from the genome sequence of Neurospora crassa: tracing the path from genomic blueprint to multicellular organism. Microbiol Mol Biol Rev 68:1–108

Brennecke J, Aravin AA, Stark A, Dus M, Kellis M, Sachidanandam R, Hannon GJ (2007) Discrete Small RNA-generating loci as master regulators of transposon activity in Drosophila. Cell 128:1089–1103

Brody H, Maiyuran S (2009) RNAi-mediated gene silencing of highly expressed genes in the industrial fungi Trichoderma reesei and Aspergillus niger. Ind Biotechnol 5:53–60

Carroll D (2011) Genome engineering with zinc-finger nucleases. Genetics 188:773–782

Cermak T, Doyle EL, Christian M, Wang L, Zhang Y, Schmidt C, Baller JA, Somia NV, Bogdanove AJ, Voytas DF (2011) Efficient design and assembly of custom TALEN and other TAL effector-based constructs for DNA targeting. Nucleic Acids Res 39(12):e82

Chang S-S, Zhang Z, Liu Y (2012) RNA interference pathways in fungi: mechanisms and functions. Annu Rev Microbiol 66:305–323

Christian M, Cermak T, Doyle EL, Schmidt C, Zhang F, Hummel A, Bogdanove AJ, Voytas DF (2010) Targeting DNA double-strand breaks with TAL effector nucleases. Genetics 186:757–761

Ciudad T, Andaluz E, Steinberg-Neifach O, Lue NF, Gow NA, Calderone RA, Larriba G (2004) Homologous recombination in *Candida albicans*: role of CaRad52p in DNA repair, integration of linear DNA fragments and telomere length. Mol Microbiol 53:1177–1194

Collemare J, Pianfetti M, Houlle AE, Morin D, Camborde L, Gagey MJ, Barbisan C, Fudal I, Lebrun MH, Bohnert HU (2008) Magnaporthe grisea avirulence gene ACE1 belongs to an infection-specific gene cluster involved in secondary metabolism. New Phytol 179:196–208

de Groot MJ, Bundock P, Hooykaas PJ, Beijersbergen AG (1998) Agrobacterium tumefaciens-mediated transformation of filamentous fungi. Nat Biotechnol 16:839–842

Djonović S, Vargas WA, Kolomiets MV, Horndeski M, Wiest A, Kenerley CM (2007) A Proteinaceous Elicitor Sm1 from the Beneficial Fungus Trichoderma virens Is Required for Induced Systemic Resistance in Maize. Plant Physiology 145:875–889

Doehlemann G, van der Linde K, Aßmann D, Schwammbach D, Hof A, Mohanty A, Jackson D, Kahmann R (2009) Pep1, a secreted effector protein of *Ustilago maydis*, is required for successful invasion of plant cells. PLoS Pathog 5:e1000290

dos Santos AC, Sena J, Santos S, Dias C, Pirovani C, Pungartnik C, Valle R, Cascardo J, Vincentz M (2009) dsRNA-induced gene silencing in Moniliophthora perniciosa, the causal agent of witches' broom disease of cacao. Fungal Genet Biol 46:825–836

Dujon B (1996) The yeast genome project: what did we learn? Trends Genet 12:263–270

Fisher MC, Henk DA, Briggs CJ, Brownstein JS, Madoff LC, McCraw SL, Gurr SJ (2012) Emerging fungal threats to animal, plant and ecosystem health. Nature 484:186–194

Frandsen RJN (2011) A guide to binary vectors and strategies for targeted genome modification in fungi using Agrobacterium tumefaciens-mediated transformation. J Microbiol Methods 87:247–262

Gardiner DM, Howlett BJ (2004) Negative selection using thymidine kinase increases the efficiency of recovery of transformants with targeted genes in the filamentous fungus Leptosphaeria maculans. Curr Genet 45:249–255

Ghildiyal M, Zamore PD (2009) Small silencing RNAs: an expanding universe. Nat Rev Genet 10:94–108

Grigoriev IV, Cullen D, Goodwin SB, Hibbett D, Jeffries TW, Kubicek CP, Kuske C, Magnuson JK, Martin F, Spatafora JW et al (2011) Fueling the future with fungal genomics. Mycology 2:192–209

Guangtao Z, Hartl L, Schuster A, Polak S, Schmoll M, Wang T, Seidl V, Seiboth B (2009) Gene targeting in a nonhomologous end joining deficient Hypocrea jecorina. J Biotechnol 139:146–151

Halic M, Moazed D (2010) Dicer-independent primal RNAs trigger RNAi and heterochromatin formation. Cell 140:504–516

He R, Guo W, Wang L, Zhang D (2015) Construction of an efficient RNAi system in the cellulolytic fungus Trichoderma reesei. J Microbiol Methods 108:70–73

Heyer W-D, Ehmsen KT, Liu J (2010) Regulation of homologous recombination in eukaryotes. Annu Rev Genet 44:113–139

Himelblau E, Gilchrist EJ, Buono K, Bizzell C, Mentzer L, Vogelzang R, Osborn T, Amasino RM, Parkin IA, Haughn GW (2009) Forward and reverse genetics of rapid-cycling *Brassica oleracea*. Theor Appl Genet 118:953–961

Horbach R, Navarro-Quesada AR, Knogge W, Deising HB (2011) When and how to kill a plant cell: infection strategies of plant pathogenic fungi. J Plant Physiol 168:51–62

James EG, Matthew RH, Li-Jun M, Christina AC, Bruce B (2005) Genomics of the fungal kingdom: insights into eukaryotic biology. Genome Res 15:1620–1631

Janus D, Hoff B, Hofmann E, Kück U (2007) An efficient fungal RNA-silencing system using the DsRed reporter gene. Appl Environ Microbiol 73:962–970

Krappmann S, Sasse C, Braus GH (2006) Gene targeting in Aspergillus fumigatus by homologous recombination is facilitated in a nonhomologous end-joining-deficient genetic background. Eukaryot Cell 5:212–215

Lau NC, Robine N, Martin R, Chung W-J, Niki Y, Berezikov E, Lai EC (2009) Abundant primary piRNAs, endo-siRNAs, and microRNAs in a Drosophila ovary cell line. Genome Res 19:1776–1785

Lawrence GJ, Dodds PN, Ellis JG (2010) TECHNICAL ADVANCE: Transformation of the flax rust fungus, Melampsora lini: selection via silencing of an avirulence gene. Plant J 61:364–369

Li X, Heyer W-D (2008) Homologous recombination in DNA repair and DNA damage tolerance. Cell Res 18:99–113

Li Z-H, Du C-M, Zhong Y-H, Wang T-H (2010) Development of a highly efficient gene targeting system allowing rapid genetic manipulations in Penicillium decumbens. Appl Microbiol Biotechnol 87:1065–1076

Magnani T, Soriani FM, de Paulo Martins V, de Freitas Policarpo AC, Sorgi CA, Faccioli LH, Curti C, Uyemura SA (2008) Silencing of mitochondrial alternative oxidase gene of Aspergillus fumigatus enhances reactive oxygen species production and killing of the fungus by macrophages. J Bioenerg Biomembr 40:631–636

Marasovic M (2015) Toward unraveling biogenesis of Dicer- independent priRNAs and siRNAs in Schizosaccharomyces pombe. Ludwig Maximilian University, Munich, p. 108

Matityahu A, Hadar Y, Dosoretz CG, Belinky PA (2008) Gene silencing by RNA Interference in the white rot fungus Phanerochaete chrysosporium. Appl Environ Microbiol 74:5359–5365

McDonald T, Brown D, Keller NP, Hammond TM (2005) RNA silencing of mycotoxin production in Aspergillus and Fusarium species. Mol Plant Microbe Interact 18:539–545

Mendoza-Mendoza A, Pozo MJ, Grzegorski D, Martínez P, García JM, Olmedo-Monfil V, Cortés C, Kenerley C, Herrera-Estrella A (2003) Enhanced biocontrol activity of Trichoderma through inactivation of a mitogen-activated protein kinase. Proc Nat Acad Sci USA 100:15965–15970

Montanari A, Bolotin-Fukuhara M, D'Orsi MF, De Luca C, Bianchi MM, Francisci S (2014) Biolistic transformation for delivering DNA. In: Genetic transformation systems in fungi, vol 1. Springer, Cham, pp 101–117

Moriwaki A, Ueno M, Arase S, Kihara J (2007) RNA-mediated gene silencing in the phytopathogenic fungus Bipolaris oryzae. FEMS Microbiol Lett 269:85–89

Mukherjee M, Mukherjee PK, Kale SP (2007) cAMP signalling is involved in growth, germination, mycoparasitism and secondary metabolism in Trichoderma virens. Microbiology 153:1734–1742

Mukherjee PK, Kenerley CM (2010) Regulation of morphogenesis and biocontrol properties in Trichoderma virens by a VELVET Protein, Vel1. Appl Environ Microbiol 76:2345–2352

Mukherjee PK, Latha J, Hadar R, Horwitz BA (2004) Role of two G-Protein alpha subunits, TgaA and TgaB, in the antagonism of plant pathogens by Trichoderma virens. Appl Environ Microbiol 70:542–549

Nayak T, Szewczyk E, Oakley CE, Osmani A, Ukil L, Murray SL, Hynes MJ, Osmani SA, Oakley BR (2006) A versatile and efficient gene-targeting system for Aspergillus nidulans. Genetics 172:1557–1566

Oliveira-Garcia E, Deising HB (2013) Infection structure–specific expression of β-1,3-glucan synthase is essential for pathogenicity of Colletotrichum oliveira graminicola and evasion of β-glucan–triggered immunity in maize. Plant Cell 25:2356–2378

Pudake RN, Swaminathan S, Sahu BB, Leandro LF, Bhattacharyya MK (2013) Investigation of the Fusarium virguliforme fvtox1 mutants revealed that the FvTox1 toxin is involved in foliar sudden death syndrome development in soybean. Curr Genet 59:107–117

Rakoczy-Trojanowska M (2002) Alternative methods of plant transformation—a short review. Cell Mol Biol Lett 7:849–858

Reh WA, Vasquez KM (2001) Gene targeting by homologous recombination. In: eLS. Wiley, Chichester

Reithner B, Brunner K, Schuhmacher R, Peissl I, Seidl V, Krska R, Zeilinger S (2005) The G protein alpha subunit Tga1 of Trichoderma atroviride is involved in chitinase formation and differential production of antifungal metabolites. Fungal Genet Biol 42:749–760

Reithner B, Schuhmacher R, Stoppacher N, Pucher M, Brunner K, Zeilinger S (2007) Signaling via the Trichoderma atroviride mitogen-activated protein kinase Tmk1 differentially affects mycoparasitism and plant protection. Fungal Genet Biol 44:1123–1133

Reyon D, Maeder ML, Khayter C, Tsai SQ, Foley JE, Sander JD, Joung JK (2013) Engineering customized TALE nucleases (TALENs) and TALE transcription factors by fast ligation-based automatable solid-phase high-throughput (FLASH) assembly. Curr Protoc Mol Biol Chapter 12:Unit 12.16

Rivera AL, Magana-Ortiz D, Gomez-Lim M, Fernandez F, Loske AM (2014) Physical methods for genetic transformation of fungi and yeast. Phys Life Rev 11:184–203

Rocha-Ramirez V, Omero C, Chet I, Horwitz BA, Herrera-Estrella A (2002) Trichoderma atroviride G-protein alpha-subunit gene tga1 is involved in mycoparasitic coiling and conidiation. Eukaryot Cell 1:594–605

Saito K, Siomi MC (2010) Small RNA-mediated quiescence of transposable elements in animals. Dev Cell 19:687–697

San Filippo J, Sung P, Klein H (2008) Mechanism of eukaryotic homologous recombination. Annu Rev Biochem 77:229–257

Sanford JC (1988) The biolistic process. Trends Biotechnol 6:299–302

Schmoll M, Franchi L, Kubicek CP (2005) Envoy, a PAS/LOV domain protein of Hypocrea jecorina (Anamorph Trichoderma reesei), modulates cellulase gene transcription in response to light. Eukaryot Cell 4:1998–2007

Schmoll M, Schuster A, Silva Rdo N, Kubicek CP (2009) The G-alpha protein GNA3 of Hypocrea jecorina (Anamorph Trichoderma reesei) regulates cellulase gene expression in the presence of light. Eukaryot Cell 8:410–420

Siomi MC, Miyoshi T, Siomi H (2010) piRNA-mediated silencing in Drosophila germlines. Semin Cell Dev Biol 21:754–759

Sioud M (2015) RNA interference: mechanisms, technical challenges, and therapeutic opportunities. Methods Mol Biol 1218:1–15

Smith M (1986) Site-directed mutagenesis. Philos Trans A Math Phys Eng Sci 317:295–304

Staats CC, Junges A, Fitarelli M, Furlaneto MC, Vainstein MH, Schrank A (2007) Gene inactivation mediated by Agrobacterium tumefaciens in the filamentous fungi Metarhizium anisopliae. Appl Microbiol Biotechnol 76:945–950

Sugui JA, Chang YC, Kwon-Chung KJ (2005) Agrobacterium tumefaciens-mediated transformation of Aspergillus fumigatus: an efficient tool for insertional mutagenesis and targeted gene disruption. Appl Environ Microbiol 71:1798–1802

Tsang A (2014) Fungal genomics. Brief Funct Genomics 13:421–423

Ulrike S, Robert M, Paul T (2015) BcNoxD, a putative ER protein, is a new component of the NADPH oxidase complex in Botrytis cinerea. Mol Microbiol 95:988–1005

Ushimaru T, Terada H, Tsuboi K, Kogou Y, Sakaguchi A, Tsuji G, Kubo Y (2010) Development of an efficient gene targeting system in Colletotrichum higginsianum using a non-homologous end-joining mutant and Agrobacterium tumefaciens-mediated gene transfer. Mol Genet Genomics 284:357–371

van Attikum H, Bundock P, Hooykaas PJJ (2001) Non-homologous end-joining proteins are required for Agrobacterium T-DNA integration. EMBO J 20:6550–6558

Vargas WA, Mukherjee PK, Laughlin D, Wiest A, Moran-Diez ME, Kenerley CM (2014) Role of gliotoxin in the symbiotic and pathogenic interactions of Trichoderma virens. Microbiology 160:2319–2330

Villalba F, Collemare J, Landraud P, Lambou K, Brozek V, Cirer B, Morin D, Bruel C, Beffa R, Lebrun MH (2008) Improved gene targeting in Magnaporthe grisea by inactivation of MgKU80 required for non-homologous end joining. Fungal Genet Biol 45:68–75

Wang W, Li W, Ma N, Steinhoff G (2013) Non-viral gene delivery methods. Curr Pharm Biotechnol 14:46–60

Wang Y, DiGuistini S, Wang T-CT, Bohlmann J, Breuil C (2010) Agrobacterium-meditated gene disruption using split-marker in Grosmannia clavigera, a mountain pine beetle associated pathogen. Curr Genet 56:297–307

Weld RJ, Plummer KM, Carpenter MA, Ridgway HJ (2006) Approaches to functional genomics in filamentous fungi. Cell Res 16:31–44

Yanagisawa S, Kikuma T, Kitamoto K (2013) Functional analysis of Aoatg1 and detection of the Cvt pathway in Aspergillus oryzae. FEMS Microbiol Lett 338:168–176

Yoshida N, Sato M (2009) Plasmid uptake by bacteria: a comparison of methods and efficiencies. Appl Microbiol Biotechnol 83:791–798

Zeilinger S, Reithner B, Scala V, Peissl I, Lorito M, Mach RL (2005) Signal transduction by Tga3, a novel G protein α subunit of Trichoderma atroviride. Appl Environ Microbiol 71:1591–1597

Chapter 6
Polyphasic Approaches to Characterize Mushroom Species

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Abstract Mushrooms are now very popular for dietary ingredients and valuable medicinal biomolecules and are continuously harvested from wild resources. For the investigation of a new species or isolates of a wild species, taxonomic identification is the first essential parameter. It is imperative that the organism is correctly identified so that the experimental data becomes valid and may be used and compared by other researchers. In different conditions, such as environmental factors, inter-hybridization and individual morphologic bias, mushroom species show a great variability in morphology and genetics, which make them difficult for the correct identification. In the present scenario, classical identification (microscopic and macroscopic comparison of morphological features) of fungi had been replaced by molecular biological techniques, based on rDNA and rRNA, which analyse and compare the ribosomal RNA (rRNA) genes from different fungal isolates. In this chapter authors have tried to explain the molecular techniques useful to characterize the mushroom species.

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6.1 Introduction

Mushrooms have been used by man for thousands of years, not only for food but also for medicinal purposes, tools, tinder, dyes, perfumes, soap, decoration and magic (Thoen 1982). The word *mushroom* may mean different things to different people and countries. It was reported (Chang and Miles 1992) that specialized studies and the economic value of mushrooms and their products had reached a point where a clear definition of the term mushroom was warranted. In a more broad sense "mushroom is a macrofungus with a distinctive fruiting body, which can be either epigeous or hypogeous and large enough to be seen with naked eye and to be picked by hand" (Chang and Miles 1992). This definition is not a perfect one but can be accepted as a workable term to estimate the number of mushrooms on the earth (Hawksworth 2001).

Mushrooms inspire awe in those encountering them. They seem different. Neither plant-like nor animal-like mushrooms have a texture, appearance and manner of growth on their own. Mushrooms represent a small branch in the evolution of the fungal kingdom *Eumycota* and are commonly known as the "fleshy fungi". Most of the edible mushrooms are basidiomycetes with the exceptions of the truffles and morels that are ascomycetes (Yun and Hall 2004). In fact, fungi are non-photosynthetic organisms that evolved from algae (Chang and Miles 2004). They include both edible/medicinal and poisonous species. However, originally, the word "mushroom" was used for the edible members of macrofungi and "toadstools" for poisonous ones of the "gill" macrofungi. Scientifically the term "toadstool" has no meaning at all, and it has been proposed that the term is dropped altogether in order to avoid confusion, and the terms edible, medicinal and poisonous mushrooms are used. Edible mushrooms once called the "food of the gods" and still treated as a garnish or delicacy can be taken regularly as part of the human diet or be treated as healthy food or as functional food. The extractable products from medicinal mushrooms, designed to supplement the human diet not as regular food but as the enhancement of health and fitness, can be classified into the category of dietary supplements/mushroom nutraceuticals (Chang and Bushwell 1996). Dietary supplements are ingredients extracted from foods, herbs, mushrooms and other plants that are taken without further modification for their presumed health-enhancing benefits.

The primary role of fungi in the ecosystem is decomposition, one organism in a succession of microbes that break down dead organic matter. And although tens of thousands of fungi are known, mushrooms constitute only a small fraction, amounting to a few thousand species.

6.2 Identification of Mushroom Species

For the investigation of a new species or isolates of a wild species, taxonomic identification is the first essential parameter. It is imperative that the organism is correctly identified so that when reference is made to published material, the experimental data becomes valid and may be used and compared by other investigators.

Here we are taking the example of polypore mushrooms. The term polypore is often used as a collective name of the group accommodating all members of *Aphyllophorales* with a poroid or tubular hymenophore. The high polymorphism of polyporoid fungi morphology complicated the creation of a natural classification of this group of fungi (Pegler 1973). The lack of unifying criteria contributed to the taxonomic controversies (Corner 1983). The latter can be well illustrated by genera *Trametes*, *Tyromyces* and *Ganoderma*. Species of these genera have a similar habitat and are often found associated with the roots or trunks of conifers and broad-leaved tree species. The genus *Ganoderma* was established by Karsten (1881) based on *G. lucidum* (W. Curt.: Fr.) P. Karst. Donk (1964) created the family *Ganodermataceae* on the basis of spore peculiarities. Different authors used diverse criteria for the taxonomy of the genus (Steyaert 1972, 1980; Murril 1915). However, the genus remains in a state of taxonomic chaos (Ryvarden 1994). Genera *Trametes* and *Tyromyces* belong to the family *Polyporaceae* and are close to the *Ganoderma* species. All three taxa are polyporoid fungi and are polymorphic in basidiocarp morphology (Pegler 1973).

In different conditions, such as environmental factors, inter-hybridization and individual morphologic bias, mushroom species show a great variability in morphology and genetics, which make them difficult for the correct identification. Besides morphological identification of species, a vast majority of techniques have been used in the laboratory for the study of genetic diversity, such as isozyme analysis (Lan et al. 1998), random amplified polymorphism DNA (RAPD) (Wang et al. 2003), amplified fragment length polymorphism (AFLP) fingerprinting, internal transcribed spacers (ITS) 25S ribosomal DNA sequencing technique (Moncalvo et al. 1995a, b) and polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) (Park et al. 1996). All these techniques identify the species at genetic level. Isozymes are generally limited by the relatively low levels of detectable polymorphism and might fail to identify cultivars, which differ in only a few genes (Jarret and Litz 1986).

The DNA sequencing of ITS1-5.8SrDNA-ITS2 is frequently used for fungal verification (Chen et al. 2003; Tuckwell et al. 2005). Although it proved to be powerful in the verification of fungal species among diverse fungal species (Tuckwell et al. 2005), it failed to discriminate variants within the strain (Zhang et al. 2003).

At the level of study, it could show that, on the basis of morphological characteristics, the identification of *G. lucidum* was not so easy. The various strains of *G. lucidum* are different both morphologically and genetically and difficult to

characterize the strainal differences because of their polyphasic approaches, habitat and climate differences such as the environmental condition, altitude and vegetation which resulted in varied colour, shape, size and other morphological traits.

Molecular markers, especially DNA techniques, are quick and reliable to establish the identities of wild collections and are helpful in mushroom taxonomy. Molecular techniques are becoming increasingly popular in the identification of new fungal species. Numerous researches have been conducted for safe identification and assessment of the genetic variability of medicinal mushrooms by use of these techniques (Luo et al. 2005).

In the present scenario classical identification (microscopic and macroscopic comparison of morphological features) of fungi had been replaced by molecular biological techniques, based on rDNA and rRNA, which analyse and compare the ribosomal RNA (rRNA) genes from different fungal isolates. These techniques include ITS, RFLP, isozyme analysis (Gottlieb et al. 1998; Gottlieb and Wright 1999; Smith and Sivasithamparam 2000) and direct sequencing of the rRNA genes (Hong et al. 2002; Moncalvo et al. 1995a, b; Smith and Sivasithamparam 2000). These techniques lead to phylogenetic analysis of species. Information about the evolutionary relationships between new and existing fungal species also analysed with the use bioinformatics tools. These techniques exhibit high sensitivity and specificity for identifying fungal strains and can be used for classifying these strains at diverse hierarchical taxonomic levels (Sette et al. 2006).

6.3 An Approach to Study Molecular Systematics of Mushrooms

The molecular data are very useful for the systematics in cases where, morphological characters are conflicting, missing, ambiguous or overlapping. Graeme Down (2002) writes about the usefulness of the molecular data in the phylogenetics of fungi – how the DNA information is converted for the evolutionary trees. RAPDs, RFLPs, AFLPs and microsatellites are the commonly used electrophoresis band pattern method. Restriction analysis of the ribosomal DNA has, of late, been extensively used in fungal taxonomy including *Ganoderma*.

PCR-based marker technologies offer the advantages of reduction in time, effort and cost involved and are hence widely used in genome characterization. RAPD technology provides researchers with a quick screen for DNA sequence-based polymorphism at a very large number of loci (Williams et al. 1990). Khush et al. (1991) first used the RAPD markers for fingerprinting the strains of *A. bisporus* and analysis of genetic variability among wild and commercial strains.

Isozymes and RFLPs were the first molecular markers used for genetic analysis in the *button mushroom*. These markers have been used for polymorphic study, identification of homokaryons and confirmation of their hybrids (Castle et al. 1988).

Amplified restriction length polymorphism (AFLP) is based on selective PCR amplification of restriction fragments generated by specific restriction enzyme (Vos et al. 1995). Microsatellites are also an important source of genetic marker due to their highly polymorphic nature and wide dispersion in the genome.

6.3.1 Internal Transcribed Spacer

The use of modern methods such as ITS analysis and direct sequencing to investigate the gene coding for the production of 16S, 5.8S, 28S and 5S rDNA has allowed assessment and comparison of phylogenetic relationship of organisms over a wide range taxonomic level. Recent studies have demonstrated that polymorphism in internal transcribed spacer (ITS) regions of 5.8S rDNA has proved adequate to improve systematic of a wide range of fungi.

The internal transcribed spacer (ITS) of nuclear ribosomal DNA (nrDNA) is a particularly valuable marker for phylogenetic analysis at interspecific and intergeneric level among fungi and other eukaryotes (Baldwin et al. 1995). The ITS regions are probably the most important regions in fungi for molecular systematics within a genus. The popularity of ITS in fungal phylogeny derived from several merits, such as biparental inheritance, universality, simplicity, intragenomic uniformity, intergenomic variability, low functional constraint and high copy number. The ITS is one of the widely accepted tool for identification of several medicinal plants and fungi, such as *G. lucidum* and also extensively applied molecular markers for fungal phylogenetic inference and genetic relatedness (Álvarez and Wendel 2003; Biffin et al. 2007; Ngan et al. 1999; Kim et al. 2007; Feng et al. 2010). It also has been successfully used as a genetic marker for molecular validation.

The highly conserved ribosomal genes, which flank the ITS regions, are ideal for universal primer targeting, and therefore the ITS regions can be amplified by PCR, and these amplified sequences can be analysed and compared and evolutionary trees can be produced. The ITS regions in fungi are highly variable, and this is useful in distinguishing between *Ganoderma* species (Moncalvo et al. 1995a, b). There have been many other workers who used this for establishing taxonomic relationships within the *Ganoderma* species (Gottlieb et al. 2000; Smith and Sivasithamparam 2000). The product of amplified ITS region can be restricted using endonucleases, and thus it in combination with ITS sequencing can further help in differentiation of variations (Zheng et al. 2009; Mir et al. 2010; Yang et al. 2010). ITS sequencing can be used to identify collections of the *G. lucidum* species complex and would help in the identification of the pathogen, in determination of host specificity and in distribution of virulent *Ganoderma* species (Hseu et al. 1996).

In contrast, the nucleotide sequence data from nuclear and mitochondrial rDNA coding regions do not offer enough variation to infer phylogenetic relationships between *Ganoderma* species (Moncalvo 2000; Roberts 2004) and therefore are only

useful at the genus level. Length of ITS PCR product is used for the identification of the species, and for *G. lucidum* it is nearly between 750–800 bp as mentioned in Moncalvo et al. (1995a, b) and Roberts (2004). Hence DNA sequencing of ITS1-5.8S rDNA-ITS2 is frequently used for fungal verification. Although it proved to be powerful in the verification of fungal species among diverse fungal species, it failed to discriminate variants within the strain (Roberts 2004).

The nuclear-encoded ribosomal RNA genes (rDNA) of fungi exist as a multiple copy gene family comprised of highly similar DNA sequences (typically from 8 to 12 kb each) arranged in a head-to-toe manner. Each repeat unit has coding regions containing the genes for the small subunit (18S), 5.8S, and large subunit (25–28S). Each unit is separated by one or more intergenic spacer (IGS) regions. In some groups (mostly basidiomycetes and some ascomycetous yeasts), each repeat also has a separately transcribed coding region for 5S RNA whose position and direction of transcription may vary among groups.

During evolution the coding regions of the 18S, 5.8S and 28S nuclear rDNA genes are highly conserved among fungi, and they show little sequence divergence between closely related species and are useful for phylogenetic studies among distantly related organisms (Roberts 2004). Within each repeat unit, the conserved regions are separated by two internal transcribed spacers, ITS I and ITS II, which show higher rates of divergence (Moncalvo et al. 1995b; Perlin and Park 2001; Roberts 2004). It is these ITS regions that are now the most widely sequenced DNA regions in fungi. Variable sequence regions in both the small (18S) and large (25S) subunits of rDNA genes have also led to numerous molecular approaches that provide rapid identification of fungal species (Perlin and Park 2001; Roberts 2004). Moncalvo et al. (1995a, b) and Smith and Sivasithamparam (2000) used rDNA-ITS sequence to distinguish between isolates of *Ganodermataceae* (Fig. 6.1).

According to Moncalvo et al. (1995a, b) who performed first time molecular taxonomy for the family *Ganodermataceae*, the controversy which has been associated with *Ganoderma* systematics in the past might be resolved with the use of molecular techniques (Roberts 2004).

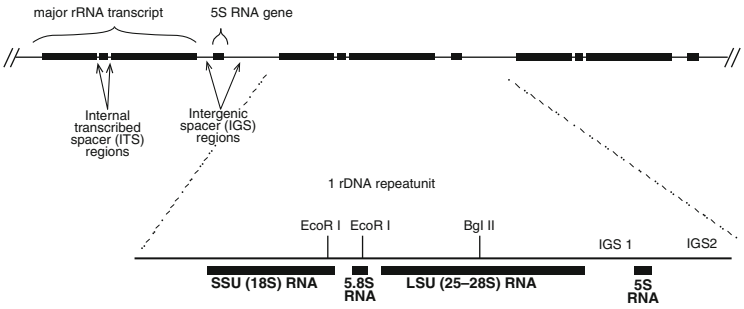

Fig. 6.1 Three rDNA genes (adapted from Vilgalys lab, Duke University (http://sites.biology.duke.edu/fungi/mycolab/primers.htm))

6.3.2 Endonuclease Restriction Digestions

There are two important techniques for digestions of DNA with restriction endonucleases, i.e. restriction fragment length polymorphism (RFLP) and random amplified polymorphic DNA (RAPD). While restriction enzyme treatment of the PCR amplified ITS regions can discriminate between most *Ganoderma* species (Park et al. 1996), RAPD can be used to differentiate between isolates having identical ITS sequences (Hseu et al. 1996).

PCR coupled with RFLP has been a useful tool in phylogenetic studies and is now widely used for fungal phylogeny and taxonomy (Hughes et al. 2002; Miller et al. 1999). PCR-RFLP was one of the first molecular approaches to approximate the differences in species. This technique, which cleaves chromosomal DNA using restriction endonucleases followed by separation of the fragments by gel electrophoresis, provides a measure of the genetics difference or relatedness between organisms (Perlin and Park 2001).

RAPD is also another useful tool in molecular biology. Park et al. (1996) analysed 28 isolates of *Ganoderma* by PCR-RFLP and RAPD and found that the resulting phenograms of genetics relatedness showed similar patterns by the two different methods, although slightly different bands were observed within the *G. lucidum* group by RAPD. Hseu et al. (1996) further attempted to differentiate isolates of the RAPD. During the RAPD analysis of *G. lucidum* complex, it was observed that groupings based on this technique did not distinguish the same clades as ITS data, and they suggested that RAPD might be helpful for systematic at the lower taxonomic level that is unresolved by ITS sequence data.

Single-stranded conformational polymorphism (SSCP) is another technique that has been used for taxonomic analysis of *Ganoderma* species. It is advantageous over restriction analysis because it is less time consuming and more economical (Gottlieb et al. 2000). In spite of being useful in determining variability, taxonomic names determined using SSCP do not correspond with the current taxonomic status of some taxa, particularly within the subgenus *Elfvingia*. These grouping were found to be almost the same as those obtained with isoenzyme data (Gottlieb et al. 1998).

6.3.3 Amplified Fragment Length Polymorphism

The AFLP technique is based on the PCR amplification of a fraction of restriction fragments generated by the digestion of total DNA (Vos et al. 1995) and has merits of RFLP and RAPD (Zabeau and Vos 1993; Vos et al. 1995; Wu et al. 2009a, b). AFLP fingerprinting was used as a tool in biodiversity studies, analysis of germplasm collection, genotyping of individuals, genetic distance analysis and genetic mapping in many kinds of organisms, including edible medicinal fungi, such as *G. lucidum* (Zheng et al. 2007), *P. ostreatus* (Meng et al. 2003), *T. matsutake* (Chen

2004), *L. edodes* (Zhuo et al. 2006) and *A. bisporus* (Gu et al. 2003; Wu et al. 2009a). The AFLP technique has a wide application prospect in molecular biology, genetics and breeding for edible medicinal mushroom. AFLP is used for the establishment of genetic database, which could provide molecular evidence for selection and breeding of eminent cultivars and genetic relationship in *Ganoderma* (Wu et al. 2009a, b).

Based on the difference of strain's fingerprint, AFLP can effectively distinguish the genotypes of different strains, which could provide effective technical means for quality control of cultivated strains and strain identification. AFLP has been successfully used in identification of main cultivated species of shiitake. Thus AFLP technique has a wide application prospect in molecular biology, genetics and breeding for mushroom (Wu et al. 2009a, b).

6.3.4 Random Amplified Polymorphic DNA

Random amplified polymorphic DNA (RAPD) analyses were used to determine the genetic relatedness within and between *Ganoderma*. RAPD are obtained by using PCR equipment. These are generated by using ten bases oligonucleotides (at least 50 % G+C) as primer for PCR amplification of genomic DNAs from different strains/species. Polymorphism is detected as the presence or absence of an amplification product. The amplified fragments are visualized under UV light following by separation on an agarose gel and staining with ethidium bromide. RAPD-PCR is one of the most sensitive and efficient methods currently available for distinguishing between different strains of a species (Hseu et al. 1996; Wu et al. 2009a; Roberts 2004). RAPD-PCR has the potential to survey entire genomes, and RAPDs may provide insights into organismal evolution that are overlooked by single-gene comparisons. Combining RAPD-PCR and sequencing methods to produce phylogenetic characters thus still may hold some promise in evolutionary genetics and systematic; by determining the nucleotide sequences of randomly amplified products, homologies between RAPDs could be inferred with greater confidence, and nucleotide sequences that are variable in homologous RAPD fragments could be used as phylogenetic characters. According to Sultmann et al. (1995), such an approach was explored recently in cichlids and showed that the RAPD-PCR technique followed by sequencing of selected fragments produced phylogenetic characters. RAPD analysis generated more variable banding patterns than PCR-RFLP analysis; this could be due to different capacities of the two analyses to reveal variations. Having the characteristics of large quantity and high sensitivity, RAPD takes the whole genome as a target and is suitable for the identification of different species. But its stability is not satisfying. Ruey-Shyang Hseu et al. (1996) studied RAPD analysis in *Agaricus bisporus* and evaluated the RAPD grouping in comparison with ITS-based phyletic groups. Because RAPD profiles are simpler and faster to produce than DNA sequences, they may present several advantages for taxonomic identification and grouping of isolates in the

G. lucidum species complex. The purpose of their study was to investigate the use of RAPD profiles in the species complex for (i) differentiation of individual strains, (ii) grouping and identification of isolates and (iii) systematics of taxonomic aggregates that were unresolved by ITS sequence data (Hseu et al. 1996; Wu et al. 2009b; Roberts 2004).

6.3.5 Restriction Fragment Length Polymorphism

It represents heritable difference in lengths of DNA fragments that are generated by digestion with restriction endonuclease. It denotes that single restriction enzyme produces fragments of different lengths from the same stretch of genomic DNA of different strains of species or from different related species. An RFLP is detected as a differential movement of a band on the gel lanes; each band is regarded as a single RFLP locus. In particular, RFLP of nuclear internal transcribed spacer (ITS) has been successfully used for taxonomy of mushrooms in conjugation with polymerase chain reaction (PCR). In this technique, the chromosomal DNA cleaves by using restriction endonuclease enzyme followed by separation of the fragments by gel electrophoresis and provides a measure of the genetic difference or relatedness between organisms (Perlin and Park 2001). PCR-RFLP has characteristics of good stability and high repeatability, which are used in phylogenetic study of microbiology. Its resolution is high in genera or genus, but the intraspecific polymorphism is not satisfying (Hseu et al. 1996; Wu et al. 2009a, b; Roberts 2004).

Research also shows that it has good resolution in different species, but it cannot reveal the difference between strains. RAPD is a more sensitive method than RFLP because RAPD resolutes at different strain levels.

6.4 Conclusion

Mushrooms are widespread with potential economic importance and show a great variability in morphology and genetics which make them difficult for their correct identification. These isolates cannot be identified by traditional taxonomic methods. Molecular markers, especially DNA techniques, are quick and reliable to establish the identities of wild collections and are helpful in mushroom taxonomy. Molecular techniques are becoming increasingly popular in the identification of new fungal species.

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References

Alvarez I, Wendel JF (2003) Ribosomal ITS sequences and plant phylogenetic inference. Mol Phylogenet Evol 29:417–434

Baldwin BG, Sanderson MJ, Porter JM, Wojciechowski MF, Campbell CS, Donoghue MJ (1995) The ITS region of nuclear ribosomal DNA: a valuable source of evidence on angiosperm phylogeny. Ann Missouri Bot Garden 82:247–277

Biffin E, Harrington MG, Crisp MD, Craven LA, Gadek PA (2007) Structural partitioning, paired-sites models and evolution of the ITS transcript in *Syzygium* and Myrtaceae. Mol Phylogenet Evol 43:124–139

Castle AJ, Horgen PA, Anderson JB (1988) Crosses among homokaryons from commercial and wild-collected strains of the mushroom *Agaricus brunnescens* (*A. bisporus*). Appl Environ Microbiol 54:1643–1648

Chang ST, Bushwell JA (1996) Mushroom nutraceutical. World J Microb Biotech 12:473–476

Chang ST, Miles PG (1992) Recent trends in world production of cultivated mushrooms. Mushroom J 203:15–18

Chang ST, Miles PG (2004) Mushrooms: cultivation, nutritional value, medicinal effect, and environmental impact, 2nd edn. CRC Press, Boca Raton, FL, p. 451

Chen AW (2004) Growing *Ganoderma* Mushroom. In: Mushroom growers handbook 1. Mush World-Heineart Inc, Seul Korea, pp 110–849

Chen Q, Liao DC, Zhang XP, Zhang CB, Li DY (2003) Preliminary analysis of genetic aspects of edible fungi *Tricholoma matsutake* isolated from Yajiang, Sichuan, by AFLP technique. Scientia Agric Sin 36:1588–1594

Corner EJH (1983) Ad polyporaceas I *Amauroderma* and *Ganoderma*. Beiheftezur Nova hedwigia 75:1–182

Donk MA (1964) A conspectus of families of Aphyllophorales. Persoonia 3:199–324

Down G (2002) Fungal family trees—finding relationships from molecular data. Mycologist 16:51–58

Feng T, Liu S, He X-J (2010) Molecular authentication of the traditional Chinese medicinal plant *Angelica sinensis* based on internal transcribed spacer of nrDNA. Electron J Biotechnol 13:1–10. doi:10.2225/vol13-issue1-fulltext-13

Gottlieb AM, Wright JE (1999) Taxonomy of *Ganoderma* from southern South America: subgenus *Ganoderma*. Mycol Res 103:661–673

Gottlieb AM, Saidman BO, Wright JE (1998) Isoenzymes of *Ganoderma* species from southern South America. Mycol Res 102:415–426

Gottlieb AM, Ferrer E, Wright JE (2000) rDNA analyses as an aid to the taxonomy of species of *Ganoderma*. Mycol Res 104:1033–1045

Gu YF, Zhang XP, Chen Q, Liao DC, Liu J (2003) Genetic variation of *Agaricus bisporus* analyzed by AFLP technique. Southwest China J Agric Sci 16:39–43

Hawksworth DL (2001) The magnitude of fungal diversity: the 1.5 million species estimate revisited. Mycol Res 105:1422–1432

Hong SG, Jeong W, Jung HS (2002) Amplification of mitochondrial small subunit ribosomal DNA of polypores and its potential for phylogenetic analysis. Mycologia 94:823–833

Hseu RS, Wang HH, Wang HF, Moncalvo JM (1996) Differentiation and grouping of isolates of the *Ganoderma lucidum* complex by random amplified polymorphic DNA-PCR compared with grouping on the basis of internal transcribed spacer sequences. Appl Environ Microbiol 62:1354–1363

Hughes CE, Bailey CD, Harris SA (2002) Divergent and reticulate species relationships in Leucaena (Fabaceae) inferred from multiple data sources: insights into polyploid origins and nrDNA polymorphism. Am J Bot 89:1057–1073

Jarret RL, Litz RE (1986) Enzyme polymorphism in *Musa acuminata* Colla. J Hered 77:183–188

Karsten P (1881) Enumeratio Boletinearumet Polyporearum Fennicarum, Systemate novo dispositarum. Rev Mycol (volume) 3:16–19

Khush RS, Morgan L, Becker E, Wach M (1991) Use of the polymerase chain reaction (PCR) in *A. bisporus* breeding programs. In: Van Griensven LJLD (ed) Genetics and breeding of Agaricus. Pudoc, Wageningen

Kim OT, Bang KH, In DS, Lee JW, Kim YC, Shin YS, Hyun DY, Lee SS, Cha SW, Seong NS (2007) Molecular authentication of ginseng cultivars by comparison of internal transcribed spacer and 5.8S rDNA sequences. Plant Biotechnol Rep 1:163–167

Lan J, Xu JT, Wang QY, He XX (1998) Electrophoretic studies on esterase and peroxidase isozymes in *Ganoderma* sp. Chin Pharm J 33:714–715

Luo LZ, Lin SQ, Xie BG, Lin ZB (2005) DNA fingerprinting analysis of *Ganoderma* strains. Acta Edulis Fungi 12:7–13

Meng Y, Jiang CS, Liao WT, Zhang YZ (2003) AFLP fingerprinting map analysis of *Pleurotus ostreatus*. Acta Genetica Sin 30:1140–1146

Miller RE, Rausher MD, Manos PS (1999) Phylogenetic systematics of Ipomoea (Convolvulaceae) based on ITS and waxy sequences. Syst Bot 24:209–227

Mir BA, Koul S, Kumar A, Koul MK, Soodan AS, Raina SN (2010) Intraspecific variation in the internal transcribed sequence (ITS) regions of rDNA in Withania somnifera. Indian J Biotechnol 9:325–328

Moncalvo JM (2000) Systematics of *Ganoderma*. In: Flood J, Bridge PD, Holderness M (eds) Ganoderma diseases of perennial crops. CABI Bioscience, Egham, pp 23–45

Moncalvo JM, Wang HF, Hseu RS (1995a) Gene phylogeny of the *Ganoderma lucidum* complex based on ribosomal DNA sequences. Comparison with traditional taxonomic characters. Mycol Res 99:1489–1499

Moncalvo JM, Wang HH, Hseu RS (1995b) Phylogenetic relationships in *Ganoderma* inferred from the internal transcribed spacers and 25S ribosomal DNA sequences. Mycologia 87:223–238

Murril WA (1915) The tropical polypores. The Author, New York

Ngan F, Shaw P, But P, Wang J (1999) Molecular authentication of *Panax* species. Phytochemistry 50:787–791

Park DS, Ryu YJ, Seok SJ, Kim YS, Yoo YB, Cha DY, Sung JM (1996) Rapid PCR-RFLP: The genetic relationship analysis of *Ganoderma* spp. using the PCR-RFLP and RAPD. J Agric Sci 38:251–260

Pegler DN (1973) Basidiospore form in the British species of *Ganoderma* Karst. Kew Bull 28:352–364

Perlin DS, Park S (2001) Rapid identification of fungal pathogens: molecular approaches for the new millennium. Rev Med Microbiol 12:S13–S20

Roberts LM (2004) Australian ganoderma: identification, growth and antibacterial properties. PhD thesis. Swinburne University of Technology

Ryvarden L (1994) Can we trust morphology in *Ganoderma*? In: Buchanan PK, Hseu RS, Moncalvo JM (eds) Systematics, phytopathology and Pharmacology. 5th international mycological congress. Department of Agriculture Chemistry, National Taiwan University, Taipei, pp 19–24

Sette LD, Passarini MRZ, Delarmelina C, Salati F, Duarte MCT (2006) Molecular characterization and antimicrobial activity of endophytic fungi from coffee plants. World J Microbiol Biotechnol 22:1185–1195

Smith BJ, Sivasithamparam K (2000) Internal transcribed spacer ribosomal DNA sequence of five species of *Ganoderma* from Australia. Mycol Res 104:943–951

Steyaert RL (1972) Species of Ganoderma and related genera mainly of the Bogor and Leiden herbaria. Persoonia 7:55–118

Steyaert RL (1980) Studies of some Ganoderma species. Bulletin du Jardin Botanique national de Belgique 50:135–186

Sultmann H, Mayer WE, Figueroa F, Tichy H, Klein J (1995) Phylogenetic analysis of cichlid fishes using nuclear DNA markers. Mol Biol Evol 12:1033–1047

Thoen, D. (1982). Usages etLégendesliés aux Polypores. Note d'ethnomycologie N° 1. Bull SocMyc FR T 98 fasc 3:289–318

Tuckwell DS, Nicholson MJ, McSweeney CS, Theodorou MK, Brookman JL (2005) The rapid assignment of ruminal fungi to presumptive genera using ITS1 and ITS2 RNA secondary structures to produce group-specific fingerprints. Microbiology 151:1557–1567

Vos P, Hogers R, Bleeker M, Reijans M, Van de Lee T, Hornes M, Frijters A, Pot J, Peleman J, Kuiper M, Zabeau M (1995) AFLP: a new technique for DNA fingerprinting. Nucl Acids Res 23:4407–4414

Wang, K., Wu, G. and Dai, S. (2003). Study on the immunological effect of the aqueous extract from Guangxi *Ganoderma lucidum* in the mice. J Guangxi Med Univ:871–874

Williams JGK, Kubelik AR, Livak KJ, Rafalski JA, Tingey SV (1990) DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Res 18:6531–6535

Wu S, Guo X, Zhou X, Li X, Chen Y, Lin J (2009a) AFLP analysis of genetic diversity in main cultivated strains of *Ganoderma* spp. Afr J Biotechnol 8:3448–3345

Wu Z, Feng J, Chen J, Xu S, Dai Z (2009b) Phylogenetic analysis and species identification of popular shrimp species in southeast China using the first internally transcribed spacer of ribosomal DNA. Aquac Res 40:1251–1259

Yang P, Zheng H, Larson S, Miao Y, Hu T (2010) Phylogenetic relationships of eleven *Kobresia* accessions from the Tibetan plateau. Afr J Biotechnol 9:3359–3367

Yun W, Hall IR (2004) Edible ectomycorrhizal mushrooms: challenges and achievements. Can J Bot 82:1063–1073

Zabeau M, Vos P (1993) Selective restriction fragment amplification: a general method for DNA fingerprinting. Publication 0 534 858 A1, bulletin 93/13. European Patent Office, Munich

Zhang JX, Xu T, Ge QX (2003) Notes on *Pestalotiopsis* from Southern China. Mycotaxon 85:91–92

Zheng LY, Jia DH, Luo X, Yang ZR (2007) AFLP analysis for genetic diversity of *Ganoderma*. China J Chin Materia Med 32:1733–1736

Zheng HM, Hu TM, Wang QZ, Zhang GY, Song JH (2009) Research of genetic diversity in seven kobresia by AFLP in tibetan plateau. Agric Sci China 8:994–999

Zhuo Y, Tan Q, Chen MJ, Cao H, Jia YN, Pan YJ (2006) AFLP analysis of genetic diversity in main cultivated strains of *Lentinula edodes*. Mycosystema 25:203–210

Chapter 7
Function-Based Metagenomics to Reveal Rhizosphere Microbiome: A Glimpse

Devendra K. Choudhary and Ajit Varma

Abstract To unravel the perplexity of cultivation methodologies, several technical efforts that involve molecular methods have been widely introduced. However, it is not feasible to elucidate the wide presence of soil microorganisms employing traditional approaches. Nowadays, a different approach has been employed, the so-called metagenome (MG), to know the functionality of bulk and rhizospheric soil, and later the result of comparative analysis on environmental MG was reported and suggested that comparative MG approach can be an extremely valuable tool for the characterization of complex microbial communities.

7.1 Introduction

Plant is a sessile organism which holds belowground with the help of roots per se, and upon seed maturity the spermosphere becomes a rhizosphere (RS). An area so-called RS is very close to the root with the range 1–10 mm and considered as a functional niche (Fig. 7.1) (Hiltner 1904). The plant rhizosphere exhibits similarity with animal intestine wherein root hairs and villus of intestine enhance the surface area of cells that helps in the nutrient uptake. Besides, in both areas, a large number of microorganisms play key roles in the decomposition of complex substances into simpler ones along with the production of vitamins and hormone-like compounds. The RS shows functional niche wherein quorum of microbes present and produce benign/detrimental secretion known as the RS effect. It has been reported that more than 99 % of microbial species present in soil are still refractory to cultivate (Amann 1995).

To unravel the perplexity of cultivation methodologies, several technical efforts that involve molecular methods have been widely introduced. However, it is not feasible to elucidate the wide presence of soil microorganisms employing

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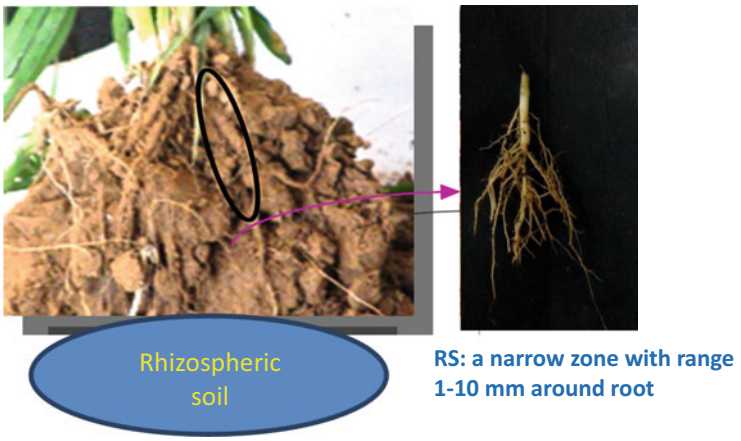

Fig. 7.1 Pictorial of RS

traditional approaches. Nowadays, a different approach has been employed, the so-called metagenome (MG), to know the functionality of soil and RS, and later the result of comparative analysis on environmental MG was reported and suggested that comparative MG approach can be an extremely valuable tool for the characterization of complex microbial communities (Edwards et al. 2006; Wegley et al. 2007; Krause et al. 2008; Schlüter et al. 2008; Diaz et al. 2009; Kröber et al. 2009).

For cultivation-independent analyses and exploitation of microbial communities present in complex ecosystems, MG has paved the way for characterization of microbes present in an ecosystem. In recent years, significant progress has been made in the deployment of MG wherein MG has been proven to be a powerful tool for the recovery of novel biomolecules. In most cases, functional MG comprising construction and screening of complex MG DNA libraries has been applied to isolate new enzymes and drugs of industrial importance. The developed MG technologies have been employed to replace culture-based approaches and allow the assessment and exploitation of the taxonomic and metabolic diversity of microbial communities on an ecosystem level (Handelsman 2004).

7.2 Function-Based MG and Rhizosphere

The sequence-based MG has been used to characterize members of gene families wherein target genes are identified either by employing PCR-based or hybridization-based approaches and probes derived from conserved regions of known genes and gene products (Daniel 2005). It is not selective for full-length genes and functional gene products, and the advantage includes the independence on gene expression and production of foreign genes in the library host (Lorenz et al. 2002). Function-driven screening of MG libraries is free of sequence information/

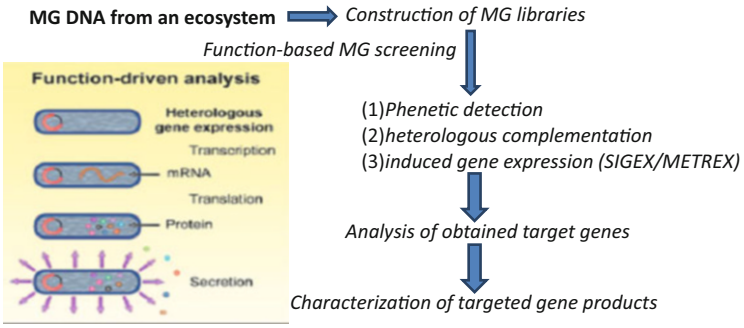

Fig. 7.2 Deployment of function-based MG approach

similarity to exist genes, and this is the approach that bears the potential to explore novel classes of functional genes (Heath et al. 2009). A major stringent of this technique is the use of foreign host *Escherichia coli* on the expression of the target genes and production of functional gene products. Hence, in function-based screening MG approach, only full-length genes and functional gene products have been detected. The three types of function-driven MG approaches have been employed to libraries that involve (1) detection of definite phenotypes of individual clones, (2) heterologous complementation of host strains and/or mutants and (3) induced gene expression (Fig. 7.2 and Table 7.1).

The functional MG libraries have been characterized by various researchers to identify enzymatic functions of individual clones by adding different substrates into the growth medium (Ferrer et al. 2009). This approach involves the detection of recombinant *E. coli* clones that showed protease activity in medium containing skimmed milk as protease substrate (Waschkowitz et al. 2009) and lipolytic activity in solid medium containing tributyrin/tricapryl as enzyme substrates (Heath et al. 2009). A varied MG approach has been used for host strains that require heterologous complementation by foreign genes for growth under selective conditions wherein a high selectivity of the screen is achieved. In the recent time the identification of DNA polymerase-encoding genes from metagenomics libraries derived from microbial communities present in glacier ice has been characterized (Simon et al. 2009). Furthermore, this approach has been employed for the detection of genes encoding Na^+/H^+ antiporters (Majernik et al. 2001), antibiotic resistance (Riesenfeld et al. 2004), enzymes involved in poly-hydroxybutyrate metabolism (Wang et al. 2006) and lysine racemases (Chen et al. 2009).

In the third type of function-based MG approach, Uchiyama et al. (2005) introduced a substrate-induced gene expression screening system (SIGEX) for the identification of novel catabolic genes wherein an operon-trap expression vector (a promoterless green fluorescent protein; gfp) was employed for cloning of environmental DNA. A similar screening strategy termed metabolite-regulated expression (METREX) has been developed together with fluorescence microscopy-mediated identification of positive fluorescent clones (Williamson et al. 2005). Now it is consensus that MG approach has been employed to characterize plant

Table 7.1 Function-based MG-derived biomolecules

Gene involved	Screening strategy	Source	Screened clones	References
Cyclodextrinase	Phenetic	Phagemids	200,000	Ferrer et al. (2005)
Beta-lactamase	Phenetic	Fosmids	8823	Song et al. (2005)
Quorum sensing inducer/inhibitor	METREX	BACs and fosmids	52,500 and 300	Williamson et al. (2005)
Aromatic hydrocarbon Catabolic operon fragments	SIGEX	Plasmids	152,000	Uchiyama et al. (2005)
Lysine racemase	Heterologous complementation	Plasmids	–	Chen et al. (2009)
Poly-3-hydroxybutyrate metabolism	Heterologous complementation	Cosmids	45,630	Wang et al. (2006)
Antibiotic resistance	Heterologous complementation	BACs and plasmids	28,200 and 1,158,000	Riesenfeld et al. (2004)
Na+/H+ antiporters	Heterologous complementation	Plasmids	1,480,000	Majernik et al. (2001)
DNA polymerase I	Heterologous complementation	Plasmids and fosmids	230,000 and 4000	Simon et al. (2009)
Antibiotics	Phenetic	Cosmids	–	Brady and Clardy (2004)
Xylanase	Phenetic	Phagemids	5,000,000	Lee et al. (2006)
Amidase	Heterologous complementation	Plasmids	193,000	Gabor et al. (2004)
Agarase	Phenetic	Cosmids	1532	Voget et al. (2003)
Protease	Phenetic Phenetic	Plasmids Fosmids	80,000 30,000	Waschkowitz et al. (2009) and Lee et al. (2007)
Cellulase	Phenetic Phenetic	Phagemids Cosmids	385,000 32,500	Rees et al. (2003) and Feng et al. (2007)
Esterase	Phenetic Phenetic	Fosmids Phagemids	5000 385,000	Rhee et al. (2005) and Rees et al. (2003)
Lipase/esterase	Phenetic	Plasmids	1,016,000	Henne et al. (2000)
Lipase	Phenetic Phenetic	Fosmids Cosmids	>7000 10,000	Hårdeman and Sjöling (2007) and Wei et al. (2009)

growth-promoting rhizobacteria (PGPRs) in RS. There are two steps for RS wherein MG involves in finding out the exploration of PGP genes and their products and the characterization of non-cultivable PGPRs. The MG approach has been employed to define bulk and RS soil with the same challenges (Daniel 2005). The relative low accessibility of initial sample may be the major obstruction in the construction of a MG library from RS soil DNA. To obviate this problem, a sample

should contain 1–10 g of soil recovered from 50 to 500 cm of root material (Jacobsen 2004). Researchers have developed methodologies to recover endophytic DNA for MG analysis, e.g. Jiao et al. (2006) employed enzymatic hydrolysis of plant tissues to recover DNA for cloning and deployed to the exploration of microbes for MG in the RS. The MG used for in vitro activity assays to exploit gain-of-function screenings of MG library from RS DNA, e.g. antibiosis to phytopathogens by testing whole-cell library clones. Several researchers have employed this assay to soilborne pathogens, e.g. *Erwinia*, *Xanthomonas*, *Fusarium*, *Rhizoctonia*, *Phytophthora* and *Pythium* (Emmert et al. 2004; Rangarajan et al. 2003; Chin-A-Woeng et al. 1998; Kim et al. 2006). Amongst functional attributes of PGPRs, researchers have employed MG library to characterize indole-3-acetic acid (IAA), cytokinins and their metabolites, genes for nitrogen fixation, exploration of RS exudates, 1-aminocyclopropane-1-carboxylate (ACC) deaminase, chitinase, solubilization of mineral phosphate, halotolerant cellulose and siderophore production (Glick et al. 1998; Timmusk et al. 1999; Rodriguez et al. 2000; Lee et al. 2003; Leveau and Lindow 2005; Tejera et al. 2005; Leveau et al. 2006; Voget et al. 2006).

The MG has been employed to understand the key metabolic processes that involved in soil phytic acid utilization. The MG analysis revealed changes in the relative abundance of the bacterial strains that enhance plant growth and phytic acid utilization together with gene clusters, namely, alkaline phosphatase and citrate synthase, with the phytic acid utilization status of the plant (Unno and Shinano 2013). In addition, MG approach has employed to assess microbial community composition and function in a constructed receiving surface water. Upon use of MG, it was estimated that the diversity of the microbial community of rhizosphere soil was found to be significantly greater than that of the wetland influent water. Functional analysis revealed a number of biodegradation pathways associated with 14 xenobiotic compounds that were identified in soil. Bai et al. (2014) found that the biological oxidation of Mn^{2+} in the influent water yielded insoluble Mn^{4+}, which subsequently precipitated and was incorporated into the wetland soil. These data showed that the use of MG analysis provided important new insights for the study of wetland ecosystems wherein how biologically mediated transformation used to reduce contamination of waste water (Bai et al. 2014). The MG approach was applied to investigate the bacterial diversity associated with the rice rhizosphere from a paddy field ecosystem in Kerala. Sequence analysis of 16S rRNA clones indicated a high diversity in the rhizosphere bacterial community with the majority of microbes being closely related to the *Proteobacteria*. Only a small fraction of the 16S rRNA sequences were highly similar to rRNA sequences from *Acidobacteria, Firmicutes* and *Bacteroidetes* groups. Since rhizosphere-associated microbes possess diverse metabolic capabilities and play a crucial role in plant health, knowledge on their community structure is imperative for the proper understanding of their individual roles, and metagenomics holds the promise to reveal several important questions regarding the unculturable fraction of the rhizosphere community (Arjun and Harikrishnan 2011).

7.3 Conclusions

Hence, MG approach is worthwhile to characterize functional microbial diversity in an environmental sample recovered from RS along with bulk soil with a particular function of interest.

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References

Amann RI (1995) Fluorescently labeled, ribosomal-rna-targeted oligonucleotide probes in the study of microbial ecology. Mol Ecol 4:543–553

Arjun JK, Harikrishnan K (2011) Metagenomic analysis of bacterial diversity in the rice rhizosphere soil microbiome. Biotechnol Bioinf Bioeng 2011(1):361–367

Bai Y, Liang J, Liu R, Hu C, Qu J (2014) Metagenomic analysis reveals microbial diversity and function in the rhizosphere soil of a constructed wetland. Environ Technol 35:2521–2527

Brady SF, Clardy J (2004) Palmitoylputrescine, an antibiotic isolated from the heterologous expression of DNA extracted frombromeliad tank water. J Nat Prod 67:1283–1286

Chen IC, Lin WD, Hsu SK, Thiruvengadam V, Hsu WH (2009) Isolation and characterization of a novel lysine racemase from a soil metagenomic library. Appl Environ Microbiol 75 (15):5161–5166. doi:10.1128/AEM.00074-09

Chin-A-Woeng TFC, Bloemberg GV, van der Bij AJ, van der Drift KMGF, Schripsema J, Kroon B et al (1998) Biocontrol by phenazine-1-carboxamide-producing *Pseudomonas chlororaphis* PCL1391 of tomato root rot caused by *Fusariumoxysporum* f. sp. radicislycopersici. Mol Plant-Microbe Interact 11:1069–1077

Daniel R (2005) Themetagenomics of soil. Nat Rev Microbiol 3:470–478

Diaz NN, Krause L, Goesmann A, Niehaus K, Nattkemper TW (2009) TACOA—taxonomic classification of environmental genomic fragments using a kernelized nearest neighbor approach. BMC Bioinformatics 10:56

Edwards RA, Rodriguez-Brito B, Wegley L, Haynes M, Breitbart M, Peterson DM, Saar MO, Alexander S, Alexander EC, Rohwer F (2006) Using pyrosequencing to shed light on deep mine microbial ecology. BMC Genomics 7:57. doi:10.1186/1471-2164-7-57

Emmert EAB, Klimowicz AK, Thomas MG, Handelsman J (2004) Genetics of Zwittermicin A production by *Bacillus cereus*. Appl Environ Microbiol 70:104–113

Feng Y, Duan CJ, Pang H, Mo XC, Wu CF, Yu Y, Hu YL, Wei J, Tang JL, Feng JX (2007) Cloning and identification of novel cellulose genes from uncultured microorganisms in rabbit cecum and characterization of the expressed cellulases. Appl Microbiol Biotechnol 75:319–328

Ferrer M, Beloqui A, Timmis KN, Golyshin PN (2009) Metagenomics for mining new genetic resources of microbial communities. J Mol Microbiol Biotechnol 16:109–123

Ferrer M, Golyshina OV, Chernikova TN, Khachane AN, Reyes-Duarte D, Santos VA, Strompl C, Elborough K, Jarvis G, Neef A, Yakimov MM, Timmis KN, Golyshin PN (2005) Novel hydrolase diversity retrieved from a metagenome library of bovine rumen microflora. Environ Microbiol 7:1996–2010

Gabor EM, de Vries EJ, Janssen DB (2004) Construction, characterization, and use of small-insert gene banks of DNA isolated from soil and enrichment cultures for the recovery of novel amidases. Environ Microbiol 6:948–958

Glick BR, Penrose DM, Li JP (1998) A model for the lowering of plant ethylene concentrations by plant growth-promoting bacteria. J Theor Biol 190:63–68

Handelsman J (2004) Metagenomics: application of genomics to uncultured microorganisms. Microbiol Mol Biol Rev 68:669–685

Hårdeman F, Sjöling S (2007) Metagenomic approach for the isolation of a novel low-temperature-active lipase from uncultured bacteria of marine sediment. FEMS Microbiol Ecol 59:524–534

Heath C, Hu XP, Cary C, Cowan D (2009) Isolation and characterization of a novel, low-temperature-active alkaliphilic esterase from an Antarctic desert soil metagenome. Appl Environ Microbiol 75:4657–4659

Henne A, Schmitz RA, Bömeke M, Gottschalk G, Daniel R (2000) Screening of environmental DNA libraries for the presence of genes conferring lipolytic activity on *Escherichia coli*. Appl Environ Microbiol 66:3113–3116

Hiltner L (1904) Überneuere Erfahrungen und Probleme auf dem Gebiete der BodenbakteriologieunterbesondererBerücksichtigung der Gründüngung und Brache. Arbeiten der Deutschen Landwirtschafts-Gesellschaft H 98:59–78

Jacobsen CS (2004) Purification of microbial genes from soil and rhizosphere by magnetic capture hybridization and subsequent amplification of target genes by PCR. In: Kowalchuk GA, De Bruijn FJ, Head IM, Akkermans ADL, Van Elsas JD (eds) Molecular microbial ecology manual. Kluwer, Dordrecht, pp 181–188

Jiao JY, Wang HX, Zeng Y, Shen YM (2006) Enrichment for microbes living in association with plant tissues. J Appl Microbiol 100:830–837

Kim J, Kim E, Kang Y, Choi O, Park CS, Hwang I (2006) Molecular characterization of biosynthetic genes of an antifungal compound produced by *Pseudomonas fluorescens* MC07. J Microbiol Biotechnol 16:450–456

Krause L, Diaz NN, Edwards RA, Gartemann KH, Krömeke H, Neuweger H, Pühler A, Runte KJ, Schlüter A, Stoye J, Szczepanowski R, Tauch A, Goesmann A (2008) Taxonomic composition and gene content of a methaneproducing microbial community isolated from a biogas reactor. J Biotechnol 136:91–101

Kröber M, Bekel T, Diaz NN, Goesmann A, Jaenicke S, Krause L, Miller D, Runte KJ, Viehöver P, Puhler A, Schlüter A (2009) Phylogenetic characterization of a biogas plant microbial community integrating clone library 16S-rDNA sequences and metagenome sequence data obtained by 454-pyrosequencing. J Biotechnol 142:38–49

Lee CC, Kibblewhite-Accinelli RE, Wagschal K, Robertson GH, Wong DW (2006) Cloning and characterization of a cold-active xylanase enzyme from an environmental DNA library. Extremophiles 10:295–300

Lee DG, Jeon JH, Jang MK, Kim NY, Lee JH, Lee JH, Kim SJ, Kim GD, Lee SH (2007) Screening and characterization of a novel fibrinolyticmetalloprotease from a metagenomic library. Biotechnol Lett 29:465–472

Lee ET, Lim SK, Nam DH, Khang YH, Kim SD (2003) Pyoverdin (2112) of *Pseudomonas fluorescens* 2112 inhibits *Phytophthoracapsici*, a red-pepper blight causing fungus. J Microbiol Biotechnol 13:415–421

Leveau JHJ, Lindow SE (2005) Utilization of the plant hormone indole-3-acetic acid for growth by *Pseudomonas putida* strain 1290. Appl Environ Microbiol 71:2365–2371

Leveau JHJ, Gerards S, Fritsche K, Zondag G, van Veen JA (2006) Genomic flank-sequencing of plasposon insertion sites for rapid identification of functional genes. J Microbiol Methods 66:276–285

Lorenz P, Liebeton K, Niehaus F, Eck J (2002) Screening for novel enzymes for biocatalytic processes: accessing the metagenome as a resource of novel functional sequence space. Curr Opin Biotechnol 13:572–577

Majernik A, Gottschalk G, Daniel R (2001) Screening of environmental DNA libraries for the presence of genes conferring Na+(Li+)/H+ antiporter activity on *Escherichia coli*:

characterization of the recovered genes and the corresponding gene products. J Bacteriol 183:6645–6653
Rangarajan S, Saleena LM, Vasudevan P, Nair S (2003) Biological suppression of rice diseases by *Pseudomonas* spp. under saline soil conditions. Plant Soil 251:73–82
Rees HC, Grant S, Jones B, Grant WD, Heaphy S (2003) Detecting cellulase and esterase enzyme activities encoded by novel genes present in environmental DNA libraries. Extremophiles 7:415–421
Rhee JK, Ahn DG, Kim YG, Oh JW (2005) Newthermophilic and thermostable esterase with sequence similarity to the hormonesensitive lipase family, cloned from a metagenomic library. Appl Environ Microbiol 71:817–825
Riesenfeld CS, Goodman RM, Handelsman J (2004) Uncultured soil bacteria are a reservoir of new antibiotic resistance genes. Environ Microbiol 6:981–989
Rodriguez H, Gonzalez T, Selman G (2000) Expression of a mineral phosphate solubilizing gene from *Erwiniaherbicola* in two rhizobacterial strains. J Biotechnol 84:155–161
Schlüter A, Bekel T, Diaz NN, Dondrup M, Eichenlaub R, Gartemann KH, Krahn I, Krause L, Krömeke H, Kruse O, Mussgnug JH, Neuweger H, Niehaus K, Pühler A, Runter KJ, Szczepanowski R, Tauch A, Tilker A, Viehöver P, Goesann A (2008) The metagenome of a biogas-producing microbial community of a production-scale biogas plant fermenter analysed by the 454-pyrosequencing technology. J Biotechnol 136:77–90
Simon C, Herath J, Rockstroh S, Daniel R (2009) Rapid identification of genes encoding DNA polymerases by function-based screening of metagenomic libraries derived from glacial ice. Appl Environ Microbiol 75:2964–2968
Song JS, Jeon JH, Lee JH, Jeong SH, Jeong BC, KimSJ LJH, Lee SH (2005) Molecular characterization of TEM-type beta-lactamases identified in cold-seep sediments of Edison Seamount (south of Lihir Island, Papua New Guinea). J Microbiol 43:172–178
Tejera N, Lluch C, Martinez-Toledo MV, Gonzalez-Lopez J (2005) Isolation and characterization of *Azotobacter* and *Azospirillum*strains from the sugarcane rhizosphere. Plant Soil 270:223–232
Timmusk S, Nicander B, Granhall U, Tillberg E (1999) Cytokinin production by *Paenibacilluspolymyxa*. Soil Biol Biochem 31:1847–1852
Uchiyama T, Abe T, Ikemura T, Watanabe K (2005) Substrate-induced gene-expression screening of environmental metagenome libraries for isolation of catabolic genes. Nat Biotechnol 23:88–93
Unno Y, Shinano T (2013) Metagenomic analysis of the rhizosphere soil microbiome with respect to phytic acid utilization. Microbes Environ 28:120–127
Voget S, Leggewie C, Uesbeck A, Raasch C, Jaeger KE, Streit WR (2003) Prospecting for novel biocatalysts in a soil metagenome. Appl Environ Microbiol 69:6235–6242
Voget S, Steele HL, Streit WR (2006) Characterization of a metagenome-derived halotolerantcellulase. J Biotechnol 126:26–36
Wang C, Meek DJ, Panchal P, Boruvka N, Archibald FS, Driscoll BT, Charles TC (2006) Isolation of poly-3-hydroxybutyrate metabolism genes from complex microbial communities by phenotypic complementation of bacterial mutants. Appl Environ Microbiol 72:384–391
Waschkowitz T, Rockstroh S, Daniel R (2009) Isolation and characterization of metalloproteases with a novel domain structure by construction and screening of metagenomic libraries. Appl Environ Microbiol 75:2506–2516
Wegley L, Edwards R, Rodriguez-Brito B, Liu H, Rohwer F (2007) Metagenomic analysis of the microbial community associated with the coral Poritesastreoides. Environ Microbiol 9:2707–2719
Wei P, Bai L, Song W, Hao G (2009) Characterization of two soil metagenome-derived lipases with high specificity for p-nitrophenylpalmitate. Arch Microbiol 191:233–240
Williamson LL, Borlee BR, Schloss PD, Guan C, Allen HK, Handelsman J (2005) Intracellular screen to identify metagenomic clones that induce or inhibit a quorum-sensing biosensor. Appl Environ Microbiol 71:6335–6344

Chapter 8
Biological Indicators for Soil Health: Potential for Development and Use of On-Farm Tests

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Abstract Agricultural sustainability can be supported by monitoring soil quality. Laboratory soil tests are available to assess a range of soil chemical, physical, and biological characteristics. Farmers can also monitor the state of their soil according to its general appearance and response to disturbance. Soil organisms have significant roles that contribute to the sustainability of agricultural systems. While on-farm tests for assessing the abundance and diversity of larger soil fauna (e.g., earthworms and other macrofauna) are available, on-farm tests for soil mesofauna, microfauna, and microorganisms are not commonly used. Adaptation of laboratory methods for quantifying soil mesofauna and arbuscular mycorrhizal (AM) fungi have potential for on-farm assessment by farmers. This chapter focuses on these two groups of soil organisms because of their multifunctional contributions to physical, chemical, and biological components of soil fertility. Soil processes in which soil mesofauna and AM fungi are involved include stabilization of aggregates. Several laboratory-based methods are available for quantifying soil mesofauna and AM fungi which can be adapted for use on-farm by farmers. Farmer motivation for investigation of soil health could lead to more sustainable land use if contributions of soil organisms are optimized. Existing farmer knowledge of soil chemical and physical characteristics based on off-farm soil tests and their use

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could be complemented by local knowledge of soil biological characteristics, especially those that involve soil mesofauna and AM fungi.

8.1 Introduction

The world population is increasing every year, leading to a greater demand for food (Pretty and Hine 2011). This increases the likelihood of problems such as soil degradation that present a major environmental threat to sustainability and productive capacity of cropping land (Pimentel et al. 1995). Soil health has been defined as the capacity of soil to function within land-use constraints while maintaining agricultural production (Idowu et al. 2008). Therefore, improvements in soil health can contribute to enhancing water and air quality, as well as plant and animal health (Romig et al. 1995). Soil organisms have roles in the development and maintenance of soil structure, nutrient cycling, and various interactions with roots (Lee and Pankhurst 1992). Arbuscular mycorrhizal (AM) fungi, common components of the microbial soil community, mediate soil-to-plant transfer of nutrients especially phosphorus via their extraradical hyphae in phosphorus-deficient soils (George et al. 1995). They can also contribute to retention of carbon in soil (Olsson 1999; Pinton et al. 2007; Solaiman 2014) and to enabling plants to access water under drying soil conditions (see review by Mickan 2014). Soil fauna contribute to nutrient cycling as well as to improving soil structure (Briones 2014; Six et al. 2004). However, soil mesofauna and AM fungi are rarely monitored as indicators of soil health using routine laboratory tests (Abbott and Lumley 2014; Paz-Ferreiro and Fu 2016; Romig et al. 1995).

An assessment of biological characteristics of soil with a selection of indicators is needed, but determining the effectiveness of using soil organisms as indicators is not straightforward (Doran and Zeiss 2000; Paz-Ferreiro and Fu 2016). Although it may be possible to characterize extremes of soil health, it is more difficult to discriminate among soils of intermediate status (Romig et al. 1995). Some soil health properties can be assessed by direct observation (Kilpatrick et al. 1999). For example, a healthy soil may appear well structured and contain an abundance of earthworms (Romig et al. 1995). In contrast, an unhealthy soil may be compacted and have few earthworms (Romig et al. 1995). Such characterization of soil is complemented by assessment of soil macrofauna on-farm (Lobry de Bruyn 1997). This chapter considers the practicality of including on-farm tests for soil mesofauna and AM fungi in assessments of soil health because of the significant roles these organisms make to the development and maintenance of soil structure and other components of soil fertility (Lee and Pankhurst 1992; Pinton et al. 2007).

8.2 Soil Biological Fertility

Soil biological fertility, a contributor to soil health, has been defined as the capacity of organisms living in soil to contribute to the nutritional requirements of plant and foraging animals while also maintaining the biological processes which improve or positively contribute to the physical and chemical state of the soil (Abbott and Murphy 2003). It is an essential component of agricultural systems as it influences nutrient release from organic matter during its decomposition, nutrient uptake by plants in association with mycorrhizas, and soil structure in parallel with chemical and physical soil properties (Abbott and Manning 2015).

Enhancing soil biological fertility could provide considerable benefits to agricultural soils because of its interconnection with soil chemical and soil physical fertility (Juarez et al. 2013; Siddiky et al. 2012). By focusing on establishing healthy soil conditions for plant growth with diverse microbial communities, it has been claimed that plants will be more resilient to disease (Altieri and Nicholls 2003). There is considerable potential for further investigation to identify how naturally occurring microbial communities can be managed to suppress pathogens (Podolich et al. 2015). Nutrient inputs from mineralization of soil organic matter can increase the resilience of agricultural crops to insect pests via biological processes which provide crop plants with potentially more balanced nutrition (Fang et al. 2012; Stirling et al. 2012), especially by moderating high levels of fertilizer N which can increase susceptibility to foliar pests (Altieri and Nicholls 2003; van Bruggen et al. 2015).

Soil fauna, microorganisms, roots, and organic matter interact to contribute to aggregation formation and stabilization in the soil (Rillig et al. 2015). Aggregation of soil particles physically protects soil organic matter and alters the structure of the soil microbial community by mediating the flow of water and oxygen, further influencing soil organic matter dynamics and nutrient cycling (Six et al. 2004). Earthworms (Satchell 1971) have received considerable attention in relation to soil aggregation (Spurgeon et al. 2013). They contribute to the formation of soil aggregates by burrowing and cast formation in soil and within burrows, and they redistribute nutrients, especially nitrogen, making them more available to plants deeper in the soil profile (van Groenigen et al. 2014).

According to their size, soil fauna comprise highly diverse groups (Handa et al. 2014; Sylvain and Wall 2011) which are generally categorized as (1) microfauna ($<$100 μm in width), which include protozoa and nematodes; (2) mesofauna (100–2000 μm in width), which include collembola, mites, and enchytraeids; and (3) macrofauna ($>$2000 μm in width), which include earthworms and termites. The activities of soil fauna are related to their size. Soil microfauna have limited ability to modify soil structure whereas soil mesofauna are larger and have greater capacity to influence processes involved in the formation of soil aggregates (Gregorich et al. 1997). Soil macrofauna redistribute organic residues in soil and expose a greater surface area for microbial decomposition and influence the macropore structure (Gregorich et al. 1997) and facilitate water infiltration (Neher 1999). Soil

mesofauna can regulate microfaunal and fungal populations and also contribute to fragmentation of organic residues (Gregorich et al. 1997). Feeding activities of soil fauna can regulate soil microbial community structure (Bardgett and Chan 1999) and abundance (Sackett et al. 2010). Therefore, while there is potential for soil fauna to be used as an indicator of soil quality (Six et al. 2004), soil mesofauna have generally been excluded from on-farm tests.

AM fungi are major components of the microbial community in soil. Most species of these fungi have the capacity to colonize roots of a wide range of plants, but they may differ in their effectiveness in promoting nutrient uptake by plants (Smith and Read 2008). They may also help plants withstand environmental stress including drought (e.g., Mickan 2014), salinity (e.g., Juniper and Abbott 1993), and heavy metals (e.g., Hildebrandt et al. 2007) and reduce the impact of root pathogens (e.g., Chandanie et al. 2006). It has been suggested that AM fungi account for 5–50 % of soil microbial biomass (Olsson 1999) which varies depending on the extent to which the fungi colonize roots and form hyphae in soil (Abbott and Gazey 1994). Based on the ubiquity of AM fungi in agricultural soils and their role in soil biological, chemical, and physical components of soil fertility, it has been suggested that they could have potential as a potential indicator of soil health (Abbott and Lumley 2014). Their abundance can be influenced by common agricultural practices such as fertilizer application, especially phosphorus (e.g., Smith and Smith 2011), tillage (e.g., Brito et al. 2012), and crop rotation (e.g., Reen et al. 2014). They may respond more rapidly to changing soil conditions associated with agricultural management practices than many other potential indicators of soil quality (Milton et al. 2002). Despite the difficulties in quantifying their contributions in soil (Gazey et al. 2004; Abbott and Lumley 2014), the potential of AM fungi as an indicator of soil health requires further consideration as a component of on-farm tests of soil health for use by farmers. This is because of their marked responsiveness to changes in agricultural practiceas well as their contributions to many components of soil fertility.

8.3 Biological Measurements of Soil Health

Soil quality has been described as “the capacity of a soil to function within the ecosystem boundaries and to interact positively with surrounding ecosystems,” and quantitative formula can be used for assessing soil quality that can help determine how soils responded to various management practices (Karlen et al. 2003). Factors that are used to assess biological soil quality include (1) root health assessment, (2) organic matter content, (3) beneficial nematode population, (4) parasitic nematode population, (5) potential mineralizable nitrogen, (6) decomposition rate or organic matter, (7) particulate organic matter, (8) active carbon test, (9) the weed seed bank, (10) microbial respiration rate, and (11) glomalin content (Karlen et al. 2003). In addition, measurement of microbial biomass (Brookes et al. 1985; Vance et al. 1987; Ananyeva et al. 2011) and plant disease (Chiang et al. 2014; Poole et al.

2015) can be used as indicators of the biological state of soil. Whether or not any of these factors can be used as indicators of soil health depends on the availability of well-defined baselines for each measurement according to the soil type and environmental conditions. Online facilities are widely available to provide farmers with information about healthy soils (Idowu et al. 2008; Table 8.1), with generally less attention to on-farm tests of biological characteristics for use by farmers to monitor their own soils. There are many soil health packages available for on-farm use, in addition to those available commercially. Some, but not all, include one or more measures of soil biological characteristics such as soil respiration, earthworm abundance, and larger soil animal abundance (see for example websites of the USDA Natural Resource Conservation Service (USA), Northern Rivers Soil Health Card, NSW Department of Primary Industries (Australia), Victorian Resources Online, Victorian Department of Economic Development, Jobs, Transport and Resources (Australia), Soil Health Environmental Health Card, Ministry for the Environment (New Zealand)). The emphasis in these packages is on physical and chemical components of soil health. However, the two examples included in Table 8.1 include greater emphasis on soil biological characteristics and require assessment off-farm.

The application of farmers' knowledge of soil fertility using a range of potential indicators includes crop yield, soil depth, drainage, moisture, manure requirements, water source, slope, and weed abundance (Desbiez et al. 2004). Farmers who wish to maximize the benefits of soil biological fertility rely more on strengthening the capability of the soil to contribute to ecosystem services, reducing fertilizer inputs, and choosing rotations and tillage practices that minimize soil and nutrient loss (Bardgett and McAlister 1999; Bhardwaj et al. 2011). Such systems can alter the activities of beneficial soil organisms and increase the relative reliance on soil biological processes (Abbott and Manning 2015). As well as adaptation of approaches to assessment of soil macrofauna (Gollan et al. 2013; Lobry de Bruyn 1997, 1999) for on-farm use, adaptation of methods for assessing the presence of mesofauna in soil and arbuscular mycorrhizal fungi in roots offers opportunities for on-farm monitoring of other soil organisms that have important contributions to soil health which are modified by soil management practices.

Table 8.1 Two examples of comprehensive approaches to monitoring soil health and soil quality (off-farm testing) which include a focus on soil biological fertility. There are many commercial soil-testing programs available for off-farm analysis of soil chemical, physical, and biological soil characteristics which differ in their emphasis on soil biology

Organization	Website
Cornell Soil Health (USA)	http://soilhealth.cals.cornell.edu/
Soilquality Program (Australia)	http://www.soilquality.org.au/

8.3.1 On-Farm Measurements of Soil Mesofauna

There are numerous methods available for measuring mesofauna in soil, but they primarily depend on equipment available in laboratories. However, a simple and portable Tullgren funnel apparatus can be made that is suited to on-farm use, and in combination with the use of a dissecting microscope or a digital microscope connected to a computer, it is now feasible for farmers to monitor soil mesofauna (Tables 8.2 and 8.3). This complements on-farm quantification of macrofauna (such as earthworms, spiders, beetles, and ants). The Monitoring Soil Science Program developed by SPICE at the University of Western Australia for use in schools, and adapted by Wheatbelt Natural Resource Management for use by farmers, is suitable for monitoring mesofauna on-farm (Table 8.2). Modification of the Tullgren/Berlese Funnel methods for assessing soil mesofauna is available for use by farmers with online support of the On-Farm Soil Monitoring Handbook to complement off-farm soil chemical tests (Wheatbelt Natural Resource Management, Western Australia—see Table 8.2). Over time, this can provide a platform for further discussion of soil health, adaptive management based on sharing of local knowledge, and group learning (Kilpatrick et al. 1999).

Methods for sampling and quantification of soil mesofauna were developed many years ago (Table 8.3), but recent access to digital microscopes makes them very suitable for on-farm monitoring. Careful consideration needs to be given to problems that may be associated with sampling, seasonal variation, heterogeneity, variability in effectiveness of sampling various groups of soil mesofauna, and appropriate times of sampling (Table 8.3). If these issues are addressed, there is an opportunity for on-farm monitoring of the general diversity and dynamics in abundance of soil fauna in relation to land use and the stage of development of crops, pastures, and perennial plants, in broadacre as well as horticultural environments such as orchards and vineyards. This approach complements off-farm commercial assessment of other soil biological characteristics, as well as commercial assessment of chemical and physical characteristics of soil, and contributes to building a stronger local knowledge foundation to observation and decision-making by farmers and their advisors.

Table 8.2 Examples of approaches to monitoring mesofauna and AM fungi in soil developed for on-farm use by school students and farmers in Western Australia as part of comprehensive programs for monitoring soil health

Monitoring soil mesofauna	Organization	Website
Monitoring Soil Science Program for schools in Western Australia	SPICE Program, Centre for Learning Technology, The University of Western Australia	http://www.news.uwa.edu.au/201202224365/earth-and-environment/global-soil-project-schools-takes-root/
On-Farm Soil Monitoring Handbook	Wheatbelt Natural Resource Management, Western Australia	http://www.wheatbeltnrm.org.au/what-we-do/sustainable-agriculture/farm-soil-monitoring

Table 8.3 Examples of methods for assessing soil mesofauna

Organisms assessed	Methodology	Key features of method	Reference
Assessments include soil collembola and mites	Flotation method, Tullgren/ Berlese Funnel methods, including use of different temperatures	Describes a range of methods No single method is suitable for all soils and situations. Considers seasonal variation	Macfadyen (1953)
	Macfadyen high gradient canister apparatus	Considers seasonal variation and issues related to collecting live and dead fauna	Leinaas (1978)
	Modified Tullgren Funnel	Considers heterogeneity of sites and sampling issues	Wood (1967)
	Tullgren funnels and a high-gradient apparatus and heating system	Considers recovery success among samples	Marshall (1972)

8.3.2 On-Farm Measurements of Arbuscular Mycorrhizal Fungi

There is no tradition of on-farm assessment of AM fungi in roots of agricultural plants by farmers. One reason for this is because of the potentially difficult procedures required for staining roots and the lack of access to a suitable microscope. Roots of plants differ in the ease with which their cellular cytoplasm can be cleared to facilitate the staining of AM fungal structures (hyphae, arbuscules, and vesicles) without background staining of the roots (Brundrett et al. 1984). In combination with the on-farm monitoring of soil mesofauna discussed above, an approach to monitoring AM fungi on-farm has also been developed (Table 8.2). This involves incorporation of staining methods that do not use the toxic chemicals that are traditionally used in research laboratories (Table 8.4). An advantage of on-farm monitoring of AM fungi is that local knowledge of potential effects of agricultural practices, including crop rotation, will be increased. Furthermore, there will be greater awareness of potential pitfalls of introducing nonlocal AM fungi (Schwartz et al. 2006) and the management practices that build and maintain effective communities of AM fungi (Abbott and Robson 1991).

Table 8.4 Examples of methods for assessing arbuscular mycorrhizal (AM) fungi in roots

Soil organisms assessed	Methodology	Key features of method	Reference
AM fungi in roots	Trypan Blue after clearing roots	Trypan Blue and phenol are toxic. Less toxic and nontoxic substitutions are available	Phillips and Hayman (1970)
	Ink and Vinegar	This simple method can be adapted for use by farmers on-farm	Vierheilig et al. (1998)
	Chlorazol black E	Widely applicability for a variety of different plant hosts	Brundrett et al. (1984)
	3,3′-diaminobenzidine (DAB)	New method tested in various plant roots	Kobae and Ohtomo (2015)

For more details of methods for staining roots to assess AM fungi, see: http://mycorrhizas.info/method.html, http://invam.wvu.edu/methods/mycorrhizae/staining-roots

8.4 Potential for Farmer Use of Soil Tests

Farmer motivation for investigation of soil health is generally based on a desire to improve productivity in a sustainable manner and involves an integrative assessment of soil physical, biological, and chemical components of soil fertility (Idowu et al. 2008; Lima et al. 2011). In this context, soil health is best assessed through soil properties that are sensitive to changes in management practices such as tillage, traffic patterns, crop rotation practices, cover crops, and organic matter additions that strongly influence the components of soil quality and thus crop performance (Idowu et al. 2008).

To design more appropriate research and to facilitate communication with farmers, there is a need to understand farmers' knowledge, perceptions, and assessments of soil fertility. This can be achieved using interviews with the farmers to gain insight into their knowledge of soil (Lobry de Bruyn and Abbey 2003), local methods used to assess the fertility status of a field, and perceived trends in soil fertility (Desbiez et al. 2004). An understanding of soil biological fertility within the farming community is a significant factor in the implementation of management practices that maintain and improve soil quality.

Farmer motivation for investigation of soil biological fertility has increased because changes in customer requirements contribute to farmer interest in changing their management practices. For example, the increased demand by consumers for organic food has increased the interest of some farmers in soil health and has led to reduced or more efficient use of chemical fertilizers. It is less clear whether this will lead to improved farm business efficiency (and hence profitability) and improved farm business viability (Kilpatrick et al. 1999).

Pressures for farmers to change their practices may arise from public concern about land and water management. The fear of change in government support to agriculture and associated changes in prices related to changes in consumer taste

can affect implementation of practices that improve soil biological fertility. Climate change also affects soil biological processes (Allen et al. 2011; Karlen et al. 2003). Education and training assists farmers to implement changes in their agricultural practice. A survey of 2500 farm businesses showed that greater participation in training, including farmer workshops, led to increased profitability (Kilpatrick et al. 1999). Further evidence of farmer interest in soil health is demonstrated through publications that address alternative farming practices (Romig et al. 1995). Farmers need to learn about reliability of on-farm as well as off-farm soil tests for monitoring trends in soil health and this will increase their awareness of how agricultural practices influence soil health (Lobry de Bruyn and Abbey 2003).

8.5 Conclusions

Soil organisms make multifunctional contributions to improving physical soil quality (e.g., *via* soil aggregation), chemical soil quality (e.g., *via* cycling of nutrients from organic matter), and biological soil quality (e.g., *via* avoidance of plant disease). While farmers understand the importance of soil organic matter, but they may be less aware of the roles of soil fauna and mycorrhizal fungi in the development and maintenance of soil structure and soil fertility. Therefore, development of on-farm soil tests for monitoring soil mesofauna and mycorrhizal fungi would complement existing tests such as those used to measure soil respiration and earthworm abundance. Based on the studies of how farmers learn (Kilpatrick et al. 1999), it is hypothesized that farmers could expand their own assessment of soil health to include monitoring of soil mesofauna and mycorrhizas. A more comprehensive package of methods for monitoring soil biological characteristics of soil on-farm would help farmers expand their knowledge of how the agricultural practices they use influence soil health.

References

Abbott LK, Gazey C (1994) An ecological view of the formation of VA mycorrhizas. Plant Soil 159:69–78

Abbott LK, Lumley S (2014) Assessing economic benefits of arbyscular mycorrhizal fungi as a potential indicator of soil health. In: Solaiman ZM, Abbott LK, Varma A (eds) Mycorrhizal fungi: use in sustainable agriculture and land restoration. Springer, Heidelberg, pp 17–31

Abbott LK, Manning DAC (2015) Soil health and related ecosystem services in organic agriculture. Sustain Agric Res 4:116–125

Abbott LK, Murphy DV (2003) What is soil biological fertility? In: Abbott LK, Murphy DV (eds) Soil biological fertility—a key to sustainable land use in agriculture. Kluwer Academic, Dordrecht, pp 1–15

Abbott LK, Robson AD (1991) Factors influencing the occurrence of vesicular-arbuscular mycorrhizas. Agric Ecosyst Environ 35:121–150

Allen DE, Singh BP, Dalal RC (2011) Soil health indicators under climate change: a review of current knowledge. In: Singh BP, Cowie AL, Chan KY (eds) Soil health and climate change, Soil biology, vol 29. Springer, Berlin, pp 25–45

Altieri MA, Nicholls CI (2003) Soil fertility management and insect pests: harmonizing soil and plant health in agroecosystems. Soil Tillage Res 72:203–211

Ananyeva ND, Susyan EA, Gavrilenko EG (2011) Determination of the soil microbial biomass carbon using the method of substrate induced respiration. Eurasian Soil Sci 44:1215–1221

Bardgett RD, Chan KF (1999) Experimental evidence that soil fauna enhance nutrient mineralization and plant nutrient uptake in montane grassland ecosystems. Soil Biol Biochem 31:1007–1014

Bardgett RD, McAlister E (1999) The measurement of soil fungal:bacterial biomass ratios as an indicator of ecosystem self-regulation in temperate meadow grasslands. Biol Fertil Soils 29:282–290

Bhardwaj AK, Jasrotia P, Hamilton SK, Robertson GP (2011) Ecological management of intensively cropped agro-ecosystems improves soil quality with sustained productivity. Agric Ecosyst Environ 140:419–429

Briones MJI (2014) Soil fauna and soil functions: a jigsaw puzzle. Front Environ Sci 2:Article 7. doi:10.3389/fenvs.2014.00007

Brito I, Goss MJ, De Carvalho M (2012) Effect of tillage and crop on arbuscular mycorrhiza colonization of winter wheat and triticale under Mediterranean conditions. Soil Use Manag 28:202–208

Brookes PC, Landman A, Pruden G, Jenkinson DS (1985) Chloroform fumigation and the release of soil nitrogen: a rapid direct extraction method to measure microbial biomass nitrogen in soil. Soil Biol Biochem 17:837–842

Brundrett MC, Piché Y, Peterson RL (1984) A new method for observing the morphology of vesicular-arbuscular mycorrhizae. Can J Bot 62:2128–2134

Chandanie WA, Kubota M, Hyakumachi M (2006) Interactions between plant growth promoting fungi and arbuscular mycorrhizal fungus Glomus mosseae and induction of systemic resistance to anthracnose disease in cucumber. Plant Soil 286:209–217

Chiang K-S, Liu S-C, Bock CH, Cottwald TR (2014) What interval characteristics make a good categorical disease assessment scale? Phytopathology 104:575–585

Desbiez A, Matthews R, Tripathi B, Ellis-Jones J (2004) Perceptions and assessment of soil fertility by farmers in the mid-hills of Nepal. Agric Ecosyst Environ 103:191–206

Doran WJ, Zeiss RM (2000) Soil health and sustainability: managing the biotic component of soil quality. Appl Soil Ecol 15:3–11

Fang X, You M, Barbetti M (2012) Reduced severity and impact of Fusarium wilt on strawberry by manipulation of soil pH, soil organic amendments and crop rotation. Eur J Plant Pathol 134:619–629

Gazey C, Abbott LK, Robson AD (2004) Indigenous and introduced arbuscular mycorrhizal fungi contribute to plant growth in two agricultural soils from south-western Australia. Mycorrhiza 14:355–362

George E, Marschner H, Jakobsen I (1995) Role of arbuscular mycorrhizal fungi in uptake of phosphorus and nitrogen from soil. Crit Rev Biotechnol 15:257–270

Gollan JR, Lobry de Bruyn L, Reid N, Wilkie L (2013) Monitoring the ecosystem service provided by dung beetles offers benefits over commonly used biodiversity metrics and traditional trapping method. J Nat Conservat 21:183–188

Gregorich GE, Carter RM, Doran WJ, Pankhurst CE, Dwyer ML (1997) Biological attributes of soil quality. Dev Soil Sci 25:81–113

Handa T, Aerts R, Berendse F, Berg MP, Bruder A, Butenschoen O, Chauvet E, Gessner MO, Jabiol J, Makkonen M, McKie BG, Malmqvist B, Peeters ETHM, Scheu S, Schmid B, van Ruijven J, Vos VCA, Hattenschwiler S (2014) Consequences of biodiversity loss for litter decomposition across biomes. Nature 509:218–221

Hildebrandt U, Regvar M, Bothe H (2007) Arbuscular mycorrhiza and heavy metal tolerance. Phytochemistry 68:139–146
Idowu OJ, van Es HM, Abawi GS, Wolfe DW, Ball JI, Gugino BK, Moebius BN, Schindelbeck RR, Bilgili AV (2008) Farmer-oriented assessment of soil quality using field, laboratory and VNIR spectroscopy methods. Plant Soil 307:243–253
Juarez S, Nunan N, Duda AC, Pouteau V, Chenu C (2013) Soil carbon mineralization responses to alterations of microbial diversity and soil structure. Biol Fertil Soils 49:939–948
Juniper S, Abbott LK (1993) Vesicular-arbuscular mycorrhizas and soil salinity. Mycorrhiza 4:45–57
Karlen LI, Ditzlerb AC, Andrews SS (2003) Soil quality: why and how? Geoderma 114:145–156
Kilpatrick S, Johns S, Prior MR and Hart D (1999) Managing farming: how farmers learn. RIRDC publication no. 99/74
Kobae Y, Ohtomo R (2015) An improved method for bright-field imaging of arbuscular mycorrhizal fungi in plant roots. Soil Sci Plant Nutr 62:1–4
Lee KE, Pankhurst CE (1992) Soil organisms and sustainable productivity. Aust J Soil Res 30:855–892
Leinaas HP (1978) Seasonal variation in sampling efficiency of Collembola and Protura. Oikos 31:307–312
Lima RCA, Hoogmoed WB, Brussaard L, Sacco dos Anjos F (2011) Farmers' assessment of soil quality in rice production systems. NJAS Wageningen J Life Sci 58:31–38
Lobry de Bruyn LA (1997) The status of soil macrofauna as indicators of soil health to monitor the sustainability of Australian agricultural soils. Ecol Econ 23:167–178
Lobry de Bruyn LA (1999) Ants as bioindicators of soil function in rural environments. Agric Ecosyst Environ 74:425–441
Lobry de Bruyn LA, Abbey AL (2003) Characterisation of farmers' soil sense and the implications for on-farm monitoring of soil health. Aust J Exp Agric 43:285–305
Macfadyen A (1953) Notes on methods for the extraction of small soil arthropods. J Anim Ecol 22:65–77
Marshall VG (1972) Comparison of two methods of estimating efficiency of funnel extractors for soil microarthropods. Soil Biol Biochem 4:417–426
Mickan B (2014) Mechanisms for alleviation of plant water stress involving arbuscular mycorrhizas. In: Solaiman ZM, Abbott LK, Varma A (eds) Mycorrhizal fungi: use in sustainable agriculture and land restoration, Soil biology series, vol 41. Springer, Berlin, pp 225–239
Milton N, Murphy D, Braimbridge M, Osler G, Jasper D, Abbott L (2002) Using power analysis to identify soil quality indicators. In: 17th world congress of soil science symposium no 32, paper 557, pp 1–8
Neher AD (1999) Soil community composition and ecosystem processes Comparing. agricultural ecosystems with natural ecosystems. Agroforestry Syst 45:159–185
Olsson PA (1999) Signature fatty acids provide tools for determination of thedistribution and interactions of mycorrhizal fungi in soil. FEMS Microbiol Ecol 29:303–310
Paz-Ferreiro J, Fu S (2016) Biological indices for soil quality evaluation: perspectives and limitations. Land Degrad Dev 27:14–25
Phillips JM, Hayman DS (1970) Improved procedures for clearing and staining vesicular-arbuscular mycorrhizal fungi for rapid assessment of infection. Trans Br Mycol Soc 55:158–161
Pimentel D, Harvey C, Resosudarmo R, Sinclair K, Kurz K, McNair M, Crist S, Shpritz L, Fitton L, Saffouri R, Blair R (1995) Environmental and economic costs of soil erosion and conservation benefits. Science 267:1117–1123
Pinton R, Varanini Z, Nanniperi P (2007) The rhizosphere biochemistry and organic substances at the soil-plant interface, 2 edn. CRC Press, Boca Raton, FL
Podolich O, Ardanov P, Zeats I, Pirttila AM, Kozyrovska N (2015) Reviving of the endophytic bacterial community as a putative mechanism of plant resistance. Plant Soil 388:367–377

Poole GJ, Harries M, Huberli D, Miyan S, Macleod WJ, Lawes R, Makay A (2015) Predicting cereal root disease in Western Australia using soil DNA and environmental parameters. Phytopathology 105:1069–1079
Pretty J, Hine R (2011) Reducing food poverty with sustainable agriculture: a summary of new evidence. Final report from the "SAFE-World"
Reen RA, Thompson JP, Clewett TG, Sheedy JG, Bell KL (2014) Yield response in chickpea cultivars and wheat following crop rotations affecting population densities of Pratylenchus thornei and arbuscular mycorrhizal fungi. Crop Pasture Sci 65:428–441
Rillig MC, Aguilar-Trigueros CA, Bergmann J, Verbruggen E, Veresoglou SD, Lehmann A (2015) Plant root and mycorrhizal fungal traits for understanding soil aggregation. New Phytol 205:1385–1388
Romig DE, Garylynd MJ, Harris RF, McSweeney K (1995) How farmers assess soil health and quality. J Soil Water Conservat 50:229–236
Sackett TE, Classen AT, Sanders NJ (2010) Linking soil food web structure to above- and belowground ecosystem processes: a meta-analysis. Oikos 119:1984–1992
Satchell JE (1971) Earthworms. In: Phillipson J (ed) Methods of study in quantitative soil ecology, IBP handbook no. 18. Blackwell, Oxford, pp 107–127
Schwartz MW, Hoeksema JD, Gehring CA, Johnson NC, Klironomos JN, Abbott LK, Pringle A (2006) The promise and the potential consequences of the global transport of mycorrhizal fungal inoculum. Ecol Lett 9:501–515
Siddiky MRK, Kolher J, Cosme M, MC R (2012) Soil biota effects on soil structure: interactions between arbuscular mycorrhizal fungal mycelium and collembola. Soil Biol Biochem 50:33–39
Six J, Bossuyt H, Degryze S, Denef K (2004) A history of research on the link between (micro) aggregates, soil biota, and soil organic matter dynamics. Soil Tillage Res 79:7–31
Smith SE, Read DJ (2008) Mycorrhizal symbiosis. Academic, London
Smith FA, Smith SE (2011) What is the significance of the arbuscular mycorrhizal colonisation of many economically important crop plants? Plant Soil 348:63–79
Solaiman ZM (2014) Contribution of arbuscular mycorrhizal fungi to soil carbon sequestration. In: Solaiman ZM, Abbott LK, Varma A (eds) Mycorrhizal fungi: use in sustainable agriculture and land restoration, Soil biology series, vol 41. Springer, Berlin, pp 287–296
Spurgeon DJ, Keith AM, Schmidt O, Lammertsma DR, Faber JH (2013) Land-use and land-management change: relationships with earthworm and fungi communities and soil structural properties. BMC Ecol 13:46. doi:10.1186/1472-6785-13-46
Stirling G, Smith M, Smith J, Stirling A, Hamill S (2012) organic inputs, tillage and rotation practices influence soil health and suppressiveness to soilborne pests and pathogens of ginger. Australas Plant Pathol 41:99–112
Sylvain ZA, Wall DH (2011) Linking soil biodiversity and vegetation: implications for a changing planet. Am J Bot 98:517–527
van Bruggen A, Francis I, Krag R (2015) The vicious cycle of lettuce corky root disease: effects of farming system, nitrogen fertilizer and herbicide. Plant Soil 388:110–132
Vance ED, Brookes PC, Jenkinson DS (1987) An extraction method for measuring soil microbial biomass. Soil Biol Biochem 19:703–707
van Groenigen JW, Lubbers IM, Vos HMJ, Brown GG, De Deyn GB, van Groenigen KJ (2014) Earthworms increase plant production: a meta-analysis. Sci Rep 4:6365. doi:10.1038/srep06365
Vierheilig H, Coughlan PA, Wyss U, Pice Y (1998) Ink and vinegar, a simple staining technique for arbuscular-mycorrhizal fungi. Appl Environ Microbiol 64:5004–5007
Wood TG (1967) Acari and Collembola of moorland soils from Yorkshire, England. III. The micro-arthropod communities. Oikos 18:277–292

Chapter 9
Mycotoxin Biosynthetic Pathways: A Window on the Evolutionary Relationships Among Toxigenic Fungi

Antonia Susca, Antonio Moretti, and Antonio F. Logrieco

Abstract Mycotoxin biosynthetic pathway involves many genes which are often clustered and co-expressed in particular conditions. These gene clusters also evolve rapidly and can also be transferred horizontally among species. However, the mechanisms of such mycotoxin cluster origin and assembly still remains a matter of speculation. It has also been studied that the growing number of available genome sequences now enables of predicting novel secondary metabolite clusters and taking a phylogenomic approach to the evolutionary origins of these clusters. The studies on regulatory pathways on control of fungal development and biosynthesis of natural products will open up new broad and exciting fields of applications in which the production of beneficial natural products could be enhanced and the production of those with deleterious effects could be reduced or eliminated.

9.1 Introduction

Mycotoxins are secondary metabolites (SM) produced by filamentous fungi and represent natural deleterious contaminants of food and feed products, pointing out health risk to humans and animals throughout the world, with a great negative impact on the world economy. A huge number of filamentous fungi, mostly belonging to the genera *Aspergillus*, *Fusarium*, and *Penicillium*, have been described as producers of the major mycotoxins that are subjected to regulation by several countries. There is evidence that mycotoxins, similarly to other SM, are compounds putatively helpful but not necessary for survival and whose production is presumably costly to maintain. Some compounds might play a role as virulence factors, or their presence could give a competitive advantage to the producing

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organism or enhance the survivability of spores, and therefore influence the development of the producing organism and members of the same species, perhaps enhancing the fitness of a given community/species.

Several studies have been conducted and are in progress, to better understand the molecular mechanisms of biosynthesis of mycotoxins and the relative regulatory steps controlling toxigenesis. The elucidation of the genetic mechanisms that operate in response to different environmental factors is of particular importance, since some of these responses are adaptations that allow these fungi to colonize plants leading to mycotoxin contamination of crops.

In many cases, genes needed for the production of SM are often grouped into clusters of coordinately regulated and functionally related genes, more likely because clustering facilitates transcriptional co-regulation. Experimental data evidence that secondary metabolite biosynthetic gene clusters are often conserved between organisms, suggesting that their maintenance could only be selected for, if the final natural product conferred some advantages to the producing organism, even though sometimes they could have subtle effects on the organism that are not immediately obvious, like mycotoxins.

Most of these mycotoxins and related clusters have been considered for a long time species or genus specific. Actually, changes in taxonomic concepts due to phylogenetic studies, the availability of more sensitive chemical detection, and identification technologies have resulted in a longer list of species proven to produce different mycotoxins. Furthermore, the expanding of genomic sequence availability and bionformatics has allowed also to discover some secondary metabolite gene clusters, or residual part of them, by *in silico* analyses giving evidence of many hitherto undiscovered or not expected pathways. All those new approaches for fungal characterization open a new scenario about new toxicological potential risks and fuller understanding of the organization, regulation, and expression of these genes. In addition, the often assessed absence of correlation between mycotoxin producing abilities and phylogenetic relationships led to the speculation that these clusters could be horizontally transferred as a unit to unrelated species, leading to the biosynthesis of the same mycotoxins in phylogenetically unrelated fungi (Walton 2000).

An early attempt to understanding the genomic structural variation among *Fusarium* species was observed in *F. oxysporum* and *F. solani* (previously known in teleomorphic stage as *Nectria Haematococca*) leading to the discovery of supernumerary chromosomes and their role in fungus–plant interaction (Boehm et al. 1994). Later, genome sequencing has provided the ultimate solution for understanding the variability in genome structure, including mycotoxin biosynthetic clusters.

The possibility of comparing nucleotide sequences from multiple species has provided insight into evolution of fungal secondary metabolite gene clusters.

Some clusters appear to have moved into fungal genomes by horizontal gene transfer from either prokaryotes or other fungi (Brakhage et al. 2005; Khaldi et al. 2008). A vertical inheritance could be responsible for the presence of clusters across multiple genera of fungi, and differential inheritance and/or deletion could

be responsible for a discontinuous distribution of clusters among groups of related fungi (Kroken et al. 2003; Proctor et al. 2004; Patron et al. 2007; Glenn et al. 2008). A gene duplication is considered to have contributed to formation of plant secondary metabolite biosynthetic clusters and clusters that regulate development in animals (Gierl and Frey 2001; Benderoth et al. 2006; Lemons and McGinnis 2006), due to high levels of sequence identity among some cluster genes. In addition, inter-genera comparisons indicate that gene relocation has contributed to the formation of an allantoin utilization cluster in yeast (Wong and Wolfe 2005) and some secondary metabolite gene clusters in plants (Field and Osbourn 2008) and has been proposed to contribute to the formation and growth of secondary metabolite gene clusters in filamentous fungi (Cary and Ehrlich 2006; Saikia et al. 2008). On the other hand, there is limited evidence for the contribution of either gene duplication or relocation in the formation of such clusters (Cary and Ehrlich 2006; Carbone et al. 2007).

9.2 Trichothecenes Biosynthetic Gene Cluster in *Fusarium*

Trichothecenes are among the most economically significant mycotoxins worldwide, because of their widespread occurrence in important grain crops such as barley, maize, and wheat (Council for Agricultural Science and Technology 2003), and are also an agricultural concern, due to their toxicity against plants and possible contribute to the pathogenesis of *Fusarium* on some crops (Desjardins et al. 1996; Maier et al. 2006).

The trichothecene biosynthetic gene cluster (*TRI* cluster) was firstly well characterized in *Fusarium graminearum* and *F. sporotrichioides*, two model species investigated to understand genetics and chemistry of trichothecene biosynthesis (Brown et al. 2001, 2002; Lee et al. 2002), where the core cluster consists of 12 genes, responsible for synthesis of the core trichothecene molecule and several modifications to it. Both species also have two smaller loci that encode trichothecene biosynthetic enzymes: a first locus consisting of a single acyl transferase gene, *TRI101*, that is responsible for esterification of acetate to the hydroxyl function at carbon atom 3 (C-3) of trichothecenes (Kimura et al. 1998; McCormick et al. 1999); and a second locus consisting of two genes, *TRI1*, which encodes a cytochrome P450 monooxygenase, and *TRI16*, which encodes an acyl transferase. In *F. sporotrichioides*, the *TRI1* enzyme catalyzes hydroxylation of trichothecenes at C-8 and the *TRI16* enzyme catalyzes esterification of a five-carbon carboxylic acid, isovalerate, to the C-8 oxygen (Brown et al. 2003; Meek et al. 2003; Peplow et al. 2003). Analysis of *TRI* loci in 16 species of *Fusarium* revealed that *TRI1* and *TRI101* are located in the core *TRI* cluster in four species of *Fusarium* (Proctor et al. 2009) that are members of the *F. incarnatum-equiseti* species complex (O'Donnell et al. 2009). This finding raised the question of whether the presence of *TRI1* and *TRI101* in the *TRI* cluster was the ancestral or derived state. For *TRI101*, it was possible to address this question, because functional or nonfunctional homologues

of *TRI101* are present in trichothecene-nonproducing species of *Fusarium* (Tokai et al. 2005; Kimura et al. 2003) that are relatively distantly related to the clade of trichothecene-producing species that was examined. The genomic context of *TRI101* in trichothecene-nonproducing species was the same as in trichothecene producing species in which *TRI101* was located outside the *TRI* cluster. Based on this, Proctor et al. (2009) concluded that it was more likely that the *TRI101* was located outside the cluster in the common ancestor of trichothecene-producing and nonproducing fusaria and subsequently moved into the cluster during the evolution of the *F. incarnatum-equiseti* species complex.

Because there was no evidence for *TRI1* homologues in trichothecene-nonproducing fusaria, other evidence was used to assess whether *TRI1* had moved into or out of the *TRI* cluster. Multiple strains of the *F. incarnatum-equiseti* species complex that were examined had an apparently functional copy of *TRI16* located outside of the cluster. In phylogenetic analyses, trees based on *TRI1* and *TRI16* sequences were highly correlated with one another but not with a species phylogeny inferred from housekeeping genes or with the genomic context of *TRI1*. In contrast, trees based on *TRI* cluster genes and on *TRI101* were highly correlated with the species phylogeny. Proctor et al. (2009) rationalized that the similar pattern of sequence variation exhibited by *TRI1* and *TRI16* but not by other *TRI* genes would have arisen more readily if *TRI1* and *TRI16* had originally been closely linked to one another and not to the *TRI* cluster. Thus, they concluded that both *TRI1* and *TRI101* originated outside of the *TRI* cluster and moved into it during divergence of the *F. incarnatum-equiseti* species complex from other lineages of trichothecene-producing fusaria. Relocation of *TRI1* and *TRI101* into the core *TRI* cluster of the *F. incarnatum-equiseti* species complex provided evidence for growth of a fungal secondary metabolite gene cluster by gene relocation rather than by gene duplication and subsequent divergence of preexisting cluster genes. Phylogenetic analysis of four *F. graminearum* monooxygenase genes (*TRI1*, *TRI4*, *TRI11*, and *TRI13*) involved in trichothecene biosynthesis provides a further independent support for this conclusion and showed that it is unlikely that the genes encoding them evolved directly from the same ancestral *TRI* gene (Proctor et al. 2009).

In the same study, the sequence data revealed that *TRI1* can be located within one of four distinct genetic environments (GE). GE1 occurs in eight species, including *F. graminearum* (Brown et al. 2003; McCormick et al. 2004), where *TRI1* is located between an orthologue of FGSG_00070 or *TRI16* (or a *TRI16* pseudogene) at 5′- and an orthologue of FGSG_00072 or sometimes FGSG_00073 at 3′-flanking region. GE2 occurs in *F. sporotrichioides* (Brown et al. 2003; Meek et al. 2003; Peplow et al. 2003) and *F. armeniacum*, where *TRI1* is located between *PDB1* consistently located in the 5′-flanking region and a variable gene in the 3′ (orthologue of *TRI16 or Orf1*/*orf1*). GE3 occurs only in *F. longipes*, where *TRI1* is located between a gene encoding a putative transcription factor and a cytochrome P450 in the *TRI1* 5′-flanking region, and a gene encoding a b-mannosidase-like protein is located in the 3′-flanking region. Finally, GE4 occurs in the four species that form the *F. equiseti* clade, i.e., *F. equiseti*, *F. scirpi*, *F. semitectum*, and *F. camptoceras*, where *TRI1* is located between *TRI10* and

TRI9 in the 5′-flanking region of *TRI1* and *TRI11* and *TRI13* in the 3′-flanking region. The exception to this is *F. camptoceras*, where *TRI11* and *TRI14* are located in the 3′-flanking region. A comparison of the *TRI1* phylogeny with the species phylogeny, inferred from combined sequences of five primary metabolic genes, revealed incongruencies between the two phylogenies. The lack of correlation between *TRI1* and species phylogenies is consistent with trans-species polymorphism, already described as possible in animals, plants, and fungi (Muirhead et al. 2002; Ward et al. 2002; Klein et al. 2007; Powell et al. 2007). Phylogenies inferred from DNA sequences of *TRI101* and the representative *TRI* cluster genes *TRI4*, *TRI5*, and *TRI11* were highly correlated with each other and with the species phylogeny derived from primary metabolic gene sequences. Those data provided to authors evidence for a complex evolutionary history of *TRI* loci that has included loss, non-functionalization and rearrangement of genes, as well as trans-species polymorphism.

9.3 Fumonisin Biosynthetic Gene Cluster in *Fusarium*

Fumonisins are a family of mycotoxins produced primarily by *Fusarium verticillioides* and *Fusarium proliferatum*, although other *Fusarium* species also may produce them. There are at least 28 different forms of fumonisins, most designated as A-series, B-series, C-series, and P-series. Fumonisin B_1 is the most common and economically important form, followed by B_2 and B_3 (Desjardins 2006). Maize is the most commonly contaminated crop, and fumonisins are the most common mycotoxins in maize, although these toxins can occur in other worldwide crops. Fumonisins are carcinogenic to laboratory animals, and in humans, consumption of fumonisin-contaminated foodstuff is associated with higher rates of esophageal cancer and neural tube birth defects.

Fumonisin biosynthetic gene (*FUM*) cluster has been firstly described in some species belonging to the *Fusarium fujikuroi* species complex (FFSC) such as *F. proliferatum* (Waalwijk et al. 2004) and *F. verticillioides* (Proctor et al. 2003). Moreover, the *FUM* cluster has been described also in the rare fumonisin-producing strains (FRC O-1890) of *F. oxysporum* (Proctor et al. 2008) and in the more distantly related fungus *Aspergillus niger* (Khaldi and Wolfe 2011). The *Fusarium* cluster includes 17 genes that encode biosynthetic enzymes as well as regulatory and transport proteins. The number, order, and orientation of genes within *FUM* clusters in *F. verticillioides*, *F. oxysporum*, and *F. proliferatum* were shown to be the same (Proctor et al. 2003, 2008; Waalwijk et al. 2004). However, the sequences flanking the clusters differ, indicating that the cluster is in a different genomic location in these three species. The genomic context of the *FUM* cluster was determined by Proctor et al. (2013) by sequence analysis of the DNA flanking each side of the cluster. The analyses indicated five different genomic contexts or genetic environments (GE), designated as GC1, GC2, GC3a, GC3b, and GC4. GC1 i s described for the full *FUM* cluster in *F. verticillioides* (Proctor et al. 2003) and

for the *FUM* cluster remnant in *Fusarium musae* (Van Hove et al. 2011), where *ORF20* and *ORF21*, representing a remnant of a gene most similar to *F. graminearum* gene FGSG_00274, are flanking the *FUM21* side and *ZBD1* and ZNF1 are flanking the *FUM19* side.

GC2 was observed in all African-clade species examined, where ANK1 and GAT1 are flanking the *FUM19* side and *ZBD1* and MFS1 are flanking the *FUM21* side. GC3a and GC3b were observed in American-clade species *F. anthophilum* and *F. bulbicola*, respectively, where they are similar for three genes (*CPM1*, *MFS2*, and *DOX1*) flanking the *FUM19* side and differ in the *FUM21*-flanking region: in GC3a (*F. anthophilum*), *FUM21* is flanked by genes *CPM2* and *TSP1*, and in GC3b (*F. bulbicola*), there was no evidence for genes.

GC4 was observed in *F. oxysporum* strain FRC O-1890, where there is no evidence for a full-length gene within the ~2800 bp region upstream of *FUM21* and an homologue of *CPM1* is flanking the *FUM19* side.

Furthermore, a phylogenetic discord of *FUM* and primary metabolism gene genealogies was evidenced and it coincides with differences in *FUM* cluster genomic context and was not consistent with fumonisin chemotype differences. The different kind of analyses conducted by Proctor et al. (2013) led to suggest that a combination of a variety of dynamic processes, including cluster duplication and loss (birth and death), balancing selection, shifts in functional constraint, translocation, and horizontal transfer (Ward et al. 2002; Carbone et al. 2007; Khaldi et al. 2008; Khaldi and Wolfe 2011; Slot and Rokas 2011), has shaped the evolution and current distribution of some secondary metabolite biosynthetic gene clusters and contributed to metabolic diversity in fungi.

9.4 Fumonisin Biosynthetic Gene Cluster in *Aspergillus*

Fumonisin production has been already supposed after finding of a putative fumonisin biosynthetic gene cluster (Baker 2006) and later confirmed also for *A niger* (Frisvad et al. 2007, 2011), perhaps the most important fungus used in biotechnology, and also one of the most commonly encountered fungi contaminating foods and feedstuffs.

The *FUM* genes are organized as a cluster of 17 genes in the species belonging to FFSC, though the location of the cluster differs among the species belonging to complex (Proctor et al. 2003; Waalwijk et al. 2004). In *A. niger* genome, an homologue of the *Fusarium* FUM cluster has been identified (Baker 2006; Pel et al. 2007), including homologues of 11 of the *Fusarium* genes: the polyketide synthase (fum1), hydroxylase (fum3, fum6, fum15), dehydrogenase (fum7), aminotransferase (fum8), acyl-CoA synthase (fum10), carbonyl reductase (fum13), condensation-domain protein (fum14), ABC transporter (fum19), and transcription factor (fum21) genes, and a short-chain dehydrogenase gene (designated here as *sdr1*) that is not present in the *Fusarium* cluster, whose function, if any, in fumonisin biosynthesis has not been reported (Baker 2006; Pel et al. 2007). A

homologue of the *Fusarium* FUM2 gene, responsible for hydroxylation of the fumonisin backbone at carbon atom 10 (Proctor et al. 2006), is notably absent from the *A. niger* fum cluster, consistently with production of B2, B4, and B6 (FB2, FB4, and FB6, respectively) reported in the majority of studies (Frisvad et al. 2007, 2011; Mansson et al. 2010; Noonim et al. 2009).

Since the clusters in *A. niger* and in *F. verticillioides* share only 11 of the 17 known *FUM* genes, these two types of cluster have probably had a long history of independent evolution (Khaldi and Wolfe 2011). In that study, authors considered horizontal transfer responsible for *fum* cluster origin in *A. niger*. With the recent taxonomic revision of the black aspergilli, fumonisin production has been confirmed only in *A. niger* and its phylogenetic cryptic sister species *A. welwitschiae* (formerly *A. awamori*; Perrone et al. 2011; Palumbo et al. 2013; Varga et al. 2010, 2011), with a discontinuous production ability of FB2 distributed among isolates of these two species. This variability was compared to the genetic structure of both species to understand what is the genetic basis for the differences in fumonisin production and nonproduction in *A. niger* and *A. welwitschiae* (Susca et al. 2014).

The studies on gene content and organization of the *fum* cluster in FB2-nonproducing isolates of *A. niger* and *A. welwitschiae* recovered from grapes provide evidence that the *fum* cluster in nonproducing isolates of *A. niger* includes all 11 fum genes as well as *sdr1*, whereas the cluster in nonproducing isolates of *A. welwitschiae* have undergone a large deletion. These findings combined with the comparisons of phylogenetic analyses between housekeeping and *fum* cluster genes suggest the mixtures of fumonisin-producing and nonproducing individuals arose independently in the two species. While the genetic basis for fumonisin nonproduction in isolates of *A. welwitschiae* is consistent with the absence of fum genes (Palumbo et al. 2013; Susca et al. 2014), in *A. niger* it remains unclear. Different mutations can potentially affect fumonisin production, i.e., 12-base deletion in *fum1* of ITEM 10355 and the premature stop codon in *fum10* of ITEM 12918, identified by gene sequencing (Susca et al. 2014), as well as a mutation(s) in eventual regulatory genes located outside the *fum* cluster.

In addition, genome sequence data of black aspergilli clade, consisting of at least 12 phylogenetically distinct species, including *A. brasiliensis*, *A. luchuensis*, *A. niger*, *A. tubingensis*, and *A. welwitschiae* (Hong et al. 2013), revealed similarities in gene content of the partial *FUM* cluster in these species. The *fum1* homologue from the partial *A. welwitschiae* cluster is more closely related to the homologue from the intact *A. welwitschiae* cluster than to homologues from partial clusters in other species (Susca et al. 2014).This suggests that the deletion(s) of the partial *FUM* cluster in *A. welwitschiae* occurred after this species diverged from other species with partial clusters. An alternative explanation for the similarity in gene content of partial *fum* cluster orthologues is nonrandom loss of genes in different species. The apparent nonrandom gene loss within the *Aspergillus* FUM cluster differs in that a full-length *fum1* has been retained in the partial clusters of the species examined except for *A. brasiliensis*. Evidence for similar genetic event is the nonrandom gene loss proved in the aflatoxin (*afl*) cluster among isolates of

A. flavus (Chang et al. 2005; Mauro et al. 2013; Moore et al. 2009) and in the gibberellic acid biosynthetic gene cluster among species of *Fusarium* (Bömke and Tudzynski 2009;Wiemann et al. 2013).

9.5 Aflatoxin Biosynthetic Gene Cluster in *Aspergillus*

Aflatoxins are potent carcinogens that include four major structural analogues: AFB_1, AFB_2, AFG_1, and AFG_2. The International Agency for Research on Cancer (IARC) has classified AFB_1 as a group 1 carcinogen in humans (IARC 1993). In addition to hepatocellular carcinoma, aflatoxins are associated with occasional outbreaks of acute aflatoxicosis that lead to death shortly after exposure (Azziz-Baumgartner et al. 2005). Aflatoxins are produced in a diversity of agricultural commodities including maize by several species of *Aspergillus*, but the two species of greatest concern are *A. flavus* and *A. parasiticus* (Frisvad et al. 2005).

The ability to produce aflatoxins is highly conserved in some species but variable in others. For example, 94–97 % of *A. parasiticus* strains that have been examined produce aflatoxins, whereas production in *A. flavus* is highly variable and depends on genotype, substrate, and geographic origin (Vaamonde et al. 2003; Pildain et al. 2004).

The *afl* biosynthetic gene cluster includes 25 genes (Yu et al. 2004). The gene content and organization of the cluster is highly conserved among *Aspergillus* species in section *Flavi*, which includes *A. flavus* and *A. parasiticus*. Sequence variability and deletions in various genes/regions of the *afl* cluster have also been used to assess variability in *A. flavus* (Chang et al. 2006). Moreover, differences in *afl* genes have been used to distinguish between aflatoxin-producing and nonproducing strains of *A. flavus* and *A. parasiticus*. Understanding such genetic differences is important because aflatoxin-nonproducing strains of *A. flavus* are used to control aflatoxin contamination in some crops (Dorner and Horn 2007). Recently, Gallo et al. (2012) examined a collection of aflatoxin-producing and nonproducing isolates of *A. flavus* for the presence of seven *afl* genes, two regulatory genes *aflR* and *aflS*, and the structural genes *aflD*, *aflM*, *aflO*, *aflP*, and *aflQ*. The result was the grouping of strains into four different amplification patterns. All aflatoxin-producing isolates yielded the complete set of amplification products, whereas nonproducing isolates did not yield products for three, four, or all seven genes, showing a high level of genetic variability among *A. flavus* isolates. Together, analyses of variation of the *afl* cluster in *A. flavus* reflect the genetic complexity of this species.

The genetic diversity of *A. flavus* populations collected from maize kernels in Northern Italy from 2003 to 2010 was also assessed by Mauro et al. (2013), by evaluating the presence or absence of several aflatoxin genes. Six deletion patterns of genes in aflatoxin cluster were detected. Regarding the atoxigenic isolates, some had no deletion in the cluster, some others had the entire cluster deleted, and only a single strain had a deletion pattern with only two genes amplified out of the

13 tested. Therefore, the genetic variability of aflatoxin cluster in non-aflatoxigenic isolates appears diversified and complex, but its understanding is important for the selection of safe and effective nonproducing strains potentially usable in biocontrol for limiting aflatoxin contamination.

Medina et al. (2014) studied the effects of the interaction of a_w, temperature, and elevated CO_2 on ten structural and regulatory aflatoxin biosynthetic genes' expression. Previously, in addition to temperature, Magan et al. (2011) have shown that changes of CO_2 concentration and water activity can influence the growth and mycotoxin production of some mycotoxigenic species, including *A. flavus*, especially under water stress. The data generated by Medina et al. (2014) showed that these interactions have a significant impact on gene expression stronger than on the growth of *A. flavus* and can stimulate the AFB_1 production. Therefore, the effect that the abovementioned environmental factors have on the expression of *A. flavus* aflatoxin genes is of extreme importance to accurately know the genetic variability of the occurring *A. flavus* populations in the field for both phylogenetic relatedness among fungal strains and mycotoxin gene biosynthetic pathways. This would strengthen the capability of predicting aflatoxin production according to the changes of environmental factors and would allow a better management of aflatoxin risk in the field.

In conclusion, the occurrence of AFB_1 at high levels in Europe in the years 2003–2004 and 2012–2013 underlines the fact that climate change will entail a change in the mycotoxin distribution patterns observed today. Global trade of plant products can also contribute to the spread of aflatoxigenic fungi and to the increase of diversity of local fungal populations. The study of the genetic structure of *afl* biosynthetic pathways in aflatoxigenic fungi is therefore essential for the development of strategies for the control of aflatoxin contamination. In this regard, the molecular characterization of native atoxigenic strains, acting through competitive exclusion of aflatoxin producers, with superior adaptation to a geographical region, should provide benefit of long-term displacement of toxigenic strains in maize environment.

9.6 Conclusion

Filamentous fungi synthesize many secondary metabolites, and genes involved in the same pathways are often clustered and co-expressed in particular conditions. Such secondary metabolism gene clusters evolve rapidly through multiple rearrangements, duplications, and losses, but also it has long been suspected that they can be transferred horizontally between species. Here are mentioned few concrete examples which have been described in the literature so far.

Anyway, much remains to be learned. The mechanism of such mycotoxin cluster origin and assembly still remains a matter of speculation, but, on the other side, the growing number of available genome sequences now enables us to both predict new

secondary metabolite clusters and take a phylogenomic approach to the evolutionary origins of these clusters.

The complexity of these regulatory networks, with multiple target sites and interconnections with other regulatory mechanisms, makes their full elucidation a challenging task. The understanding of the regulatory pathways on control of fungal development and biosynthesis of natural products will open up new broad and exciting fields of applications in which the production of beneficial natural products could be enhanced and the production of those with deleterious effects could be reduced or eliminated.

References

Azziz-Baumgartner E, Lindblade K, Gieseker K, Rogers HS, Kieszak S, Njapau H, Schleicher R, McCoy LF, Misore A, DeCock K, Rubin C, Slutsker L (2005) Case-control study of an acute aflatoxicosis outbreak, Kenya. Environ Health Perspect 113:1799–1783

Baker SE (2006) Aspergillus niger genomics: past, present and into the future. Med Mycol 44: S17–S21

Benderoth M, Textor S, Windsor AJ, Mitchell-Olds T, Gershenzon J, Kroymann J (2006) Positive selection driving diversification in plant secondary metabolism. Proc Natl Acad Sci USA 103:9118–9123

Boehm EWA, Plotetz RC, Kistler HC (1994) Statistical analysis of electrophoretic karyotype variation among vegetative compatibility groups of *Fusarium oxysporum f. sp. cubense*. Mol Plant Microbe Interact 7:196–207

Bömke C, Tudzynski B (2009) Diversity, regulation, and evolution of the gibberellin biosynthetic pathway in fungi compared to plants and bacteria. Phytochemistry 70:1876–1893

Brakhage AA, Al-Abdallah Q, Tüncher A, Spröte P (2005) Evolution of b-lactam biosynthesis and recruitment of trans-acting factors. Phytochemistry 66:1200–1210

Brown DW, McCormick SP, Alexander NJ, Proctor RH, Desjardins AE (2001) A genetic and biochemical approach to study trichothecene diversity in *Fusarium sporotrichioides* and *Fusarium graminearum*. Fungal Genet Biol 32:121–133

Brown DW, McCormick SP, Alexander NJ, Proctor RH, Desjardins AE (2002) Inactivation of a cytochrome P-450 is a determinant of trichothecene diversity in *Fusarium* species. Fungal Genet Biol 36:224–233

Brown DW, Proctor RH, Dyer RB, Plattner RD (2003) Characterization of a *Fusarium* 2-gene cluster involved in trichothecene C-8 modification. J Agric Food Chem 51:7936–7944

Carbone I, Ramirez-Prado JH, Jakobek JL, Horn BW (2007) Gene duplication, modularity and adaptation in the evolution of the aflatoxin gene cluster. BMC Evol Biol 7:111. doi:10.1186/1471-2148-7-111

Cary JW, Ehrlich KC (2006) Aflatoxigenicity in Aspergillus: molecular genetics, phylogenetic relationships and evolutionary implications. Mycopathologia 162:167–177

CAST—Council for Agricultural Science and Technology (2003) Mycotoxins: risks in plant, animal, and human systems. Task Force Report No. 139. Council for Agricultural Science and Technology, Ames, IA

Chang PK, Horn BW, Dorner JW (2005) Sequence breakpoints in the aflatoxin biosynthesis gene cluster and flanking regions in nonaflatoxigenic Aspergillus flavus isolates. Fungal Genet Biol 42:914–923

Chang PK, Ehrlich KC, Hua SST (2006) Cladal relatedness among *Aspergillus oryzae* isolates and *Aspergillus flavus* S and L morphotype isolates. Int J Food Microbiol 108:172–177

Desjardins AE (2006) Fusarium mycotoxins: chemistry, genetics, and biology. APS Press, St. Paul, MN 260p

Desjardins AE, Proctor RH, Bai G, McCormick SP, Shaner G, Buechley G, Hohn TM (1996) Reduced virulence of trichothecene-nonproducing mutants of *Gibberella zeae* in wheat field tests. Mol Plant Microbe Interact 9:775–781

Dorner JW, Horn BW (2007) Separate and combined applications of nontoxigenic *Aspergillus flavus* and *A. parasiticus* for biocontrol of aflatoxin in peanuts. Mycopathologia 163:215–223

Field B, Osbourn AE (2008) Metabolic diversification—independent assembly of operon-like gene clusters in different plants. Science 320:543–547

Frisvad JC, Skouboe P, Samson RA (2005) Taxonomic comparison of three different groups of aflatoxin producers and a new efficient producer of aflatoxin B1, sterigmatocystin and 3-O-methyl sterigmatocystin, *Aspergillus rambellii* sp. nov. Syst Appl Microbiol 28:442–453

Frisvad JC, Smedsgaard J, Samson RA, Larsen TO, Thrane U (2007) Fumonisin B2 production by Aspergillus niger. J Agric Food Chem 55:9727–9732

Frisvad JC, Larsen TO, Thrane U, Meijer M, Varga J, Samson RA (2011) Fumonisin and ochratoxin production in industrial *Aspergillus niger* strains. PLoS One 6(8):e23496 http://dx.doi.org/10.1371/journal.pone.0023496

Gallo A, Stea G, Battilani P, Logrieco AF, Perrone G (2012) Molecular characterization of an *Aspergillus flavus* population isolated from maize during the first outbreak of aflatoxin contamination in Italy. Phytopathologia Mediterranea 51:198–206

Gierl A, Frey M (2001) Evolution of benzoxazinone biosynthesis and indole production in maize. Planta 213:493–498

Glenn AE, Zitomer NC, Zimeri AM, Williams LD, Riley RT, Proctor RH (2008) Transformation-mediated complementation of a FUM gene cluster deletion in Fusarium verticillioides restores both fumonisin production and pathogenicity on maize seedlings. Mol Plant Microbe Interact 21:87–97

Hong SB, Lee M, Kim DH, Varga J, Frisvad JC, Perrone G, Gomi K, Yamada O, Machida M, Houbraken J, Samson RA (2013) Aspergillus luchuensis, an industrially important black Aspergillus in East Asia. PLoS One 8:e63769. doi:10.1371/journal.pone.0063769

IARC (International Agency for Cancer Research) (1993) Some naturally occurring substances: food items and constituents, heterocyclic aromatic amines and mycotoxins. IARC Monogr Eval Carcinog Risks Hum 56:1–599

Khaldi N, Wolfe KH (2011) Evolutionary origins of the fumonisin secondary metabolite gene cluster in Fusarium verticillioides and Aspergillus niger. Int J Evol Biol. doi:10.4061/2011/423821

Khaldi N, Collemare J, Lebrun M-H, Wolf KH (2008) Evidence for horizontal transfer of a secondary metabolite gene cluster between fungi. Genome Biol 9:R18.1–R18.10

Kimura M, Kaneko I, Komiyama M, Takatsuki A, Koshino H, Yoneyama K, Yamaguchi I (1998) Trichothecene 3-*O*-acetyltransferase protects both the producing organism and transformed yeast from related mycotoxins. J Biol Chem 273:1654–1661

Kimura M, Tokai T, O'Donnell K, Ward TJ, Fujimura M, Hamamoto H, Shibata T, Yamaguchi I (2003) The trichothecene biosynthesis gene cluster of *Fusarium graminearum* F15 contains a limited number of essential pathway genes and expressed non-essential genes. FEBS Lett 359:105–110

Klein J, Sato A, Nikolaidis N (2007) MHC, TSP, and the origin of species: from immunogenetics to evolutionary genetics. Annu Rev Genet 41:281–304

Kroken S, Glass NL, Taylor JW, Yoder OC, Turgeon BG (2003) Phylogenomic analysis of type I polyketide synthase genes in pathogenic and saprobic ascomycetes. Proc Natl Acad Sci USA 100:15670–15675

Lee T, Han Y-K, Kim K-H, Yun S-H, Lee Y-W (2002) *Tri13* and *Tri7* determine deoxynivalenol- and nivalenol-producing chemotypes of *Gibberella zeae*. Appl Environ Microbiol 68:2148–2154

Lemons D, McGinnis W (2006) Genomic evolution of Hox gene clusters. Science 313:1918–1922

Magan N, Medina A, Aldred D (2011) Possible climate change effects on mycotoxin contamination of food crops pre- and postharvest. Plant Pathol 60:150–163

Maier FJ, Miedaner T, Hadeler B, Felk A, Salomon S, Lemmens M et al (2006) Involvement of trichothecenes in fusarioses of wheat, barley and maize evaluated by gene disruption of the trichodiene synthase (*Tri5)* gene in three field isolates of different chemotype and virulence. Mol Plant Pathol 7:449–461

Mansson M, Klejnstrup ML, Phipps RK, Nielsen KF, Frisvad JC, Gotfredsen CH, Larsen TO (2010) Isolation and NMR characterization of fumonisin B2 and a new fumonisin B6 from Aspergillus niger. J Agric Food Chem 58:949–953

Mauro A, Battilani P, Callicott KA, Giorni P, Pietri A, Cotty PJ (2013) Structure of an Aspergillus flavus population from maize kernels in northern Italy. Int J Food Microbiol 162:1–7

McCormick SP, Alexander NJ, Trapp SC, Hohn TM (1999) Disruption of *TRI101*, the gene encoding trichothecene 3-*O*-acetyltransferase, from *Fusarium sporotrichioides*. Appl Environ Microbiol 65:5252–5256

McCormick SP, Harris LJ, Alexander NJ, Ouellet T, Saparno A, Allard S, Desjardins AE (2004) *Tri1* in *Fusarium graminearum* encodes a P450 oxygenase. Appl Environ Microbiol 70:2044–2051

Medina A, Rodriguez A, Magan N (2014) Effect climate change on *Aspergillus flavus* and aflatoxin B_1 production. Front Microbiol 5:1–7

Meek IB, Peplow AW, Ake C, Phillips TD, Beremand MN (2003) *Tri1* encodes the cytochrome P450 monooxygenase for C-8 hydroxylation during trichothecene biosynthesis in *Fusarium sporotrichioides* and resides upstream of another new *Tri* gene. Appl Environ Microbiol 69:1607–1613

Moore GG, Singh R, Horn BW, Carbone I (2009) Recombination and lineage-specific gene loss in the aflatoxin gene cluster of *Aspergillus flavus*. Mol Ecol 18:4870–4887

Muirhead CA, Glass NL, Slatkin M (2002) Multilocus self-recognition systems in fungi as a cause of trans-species polymorphism. *Genetics* 161:633–641

Noonim P, Mahakarnchanakul W, Nielsen KF, Frisvad JC, Samson RA (2009) Fumonisin B2 production by *Aspergillus niger* in Thai coffee beans. Food Addit Contam Part A Chem Anal Control Expo Risk Assess 26:94–100

O'Donnell K, Sutton DA, Rinaldi MG, Gueidan C, Crous PW, Geiser DM (2009) A novel multilocus sequence typing scheme reveals high genetic diversity of human pathogenic members of the *Fusarium incarnatum-F. equiseti* and *F. chlamydosporum* species complexes within the U.S. J Clin Microbiol 47:3851–3861

Palumbo JD, O'Keeffe TL, Gorski L (2013) Multiplex PCR analysis of fumonisin biosynthetic genes in fumonisin-nonproducing *Aspergillus niger* and *A. awamori* strains. Mycologia 105:277–284

Patron NJ, Waller RF, Cozijnsen AJ, Straney DC, Gardiner DM, Nierman WC, Howlett BJ (2007) Origin and distribution of epipolythiodioxopiperazine (ETP) gene clusters in filamentous ascomycetes. BMC Evol Biol 7:174. doi:10.1186/1471-2148-7-174

Pel HJ, de Winde JH, Archer DB, Dyer PS, Hofmann G, Schaap PJ, Turner G, de Vries RP, Albang R, Albermann K, Andersen MR, Bendtsen JD, Benen JA, van den Berg M, Breestraat S, Caddick MX, Contreras R, Cornell M, Coutinho PM, Danchin EG, Debets AJ, Dekker P, van Dijck PW, van Dijk A, Dijkhuizen L, Driessen AJ, d'Enfert C, Geysens S, Goosen C, Groot GS, de Groot PW, Guillemette T, Henrissat B, Herweijer M, van den Hombergh JP, van den Hondel CA, van der Heijden RT, van der Kaaij RM, Klis FM, Kools HJ, Kubicek CP, van Kuyk PA, Lauber J, Lu X, van der Maarel MJ, Meulenberg R, Menke H, Mortimer MA, Nielsen J, Oliver SG, Olsthoorn M, Pal K, van Peij NN, Ram AF, Rinas U, Roubos JA, Sagt CM, Schmoll M, Sun J, Ussery D, Varga J, Vervecken W, van de Vondervoort PJ, Wedler H, Wosten HA, Zeng AP, van Ooyen AJ, Visser J, Stam H (2007) Genome sequencing and analysis of the versatile cell factory *Aspergillus niger* CBS 513.88. Nat Biotechnol 25:221–231

Peplow AW, Meek IB, Wiles MC, Phillips TD, Beremand MN (2003) *Tri16* is required for esterification of position C-8 during trichothecene mycotoxin production by *Fusarium sporotrichioides*. Appl Environ Microbiol 69:5935–5940

Perrone G, Stea G, Epifani F, Varga J, Frisvad JC, Samson RA (2011) *Aspergillus niger* contains the cryptic phylogenetic species A. awamori. Fungal Biol 115:1138–1150

Pildain MB, Vaamonde G, Cabral D (2004) Analysis of population structure of *Aspergillus flavus* from peanut based on vegetative compatibility, geographic origin, mycotoxin and sclerotia production. Int J Food Microbiol 93:31–40

Powell AJ, Jacobson DJ, Natvig DO (2007) Ancestral polymorphism and linkage disequilibrium at the *het-6* region in pseudohomothallic *Neurospora tetrasperma*. Fungal Genet Biol 44:896–904

Proctor RH, Brown DW, Plattner RD, Desjardins AE (2003) Co-expression of 15 contiguous genes delineates a fumonisin biosynthetic gene cluster in Gibberella moniliformis. Fungal Genet Biol 38:237–249

Proctor RH, Plattner RD, Brown DW, Seo J-A, Lee Y-W (2004) Discontinuous distribution of fumonisin biosynthetic genes in the *Gibberella fujikuroi* species complex. Mycol Res 108:815–822

Proctor RH, Plattner RD, Desjardins AE, Busman M, Butchko RAE (2006) Fumonisin production in the maize pathogen *Fusarium verticillioides*: genetic basis of naturally occurring chemical variation. J Agric Food Chem 54:2424–2430

Proctor RH, Busman M, Seo J-A, Lee YW, Plattner RD (2008) A fumonisin biosynthetic gene cluster in Fusarium oxysporum strain O-1890 and the genetic basis for B versus C fumonisin production. Fungal Genet Biol 45:1016–1026

Proctor RH, McCormick SP, Alexander NJ, Desjardins AE (2009) Evidence that a secondary metabolic biosynthetic gene cluster has grown by gene relocation during evolution of the filamentous fungus *Fusarium*. Mol Microbiol 74:1128–1142

Proctor RH, Van Hove F, Susca A, Stea G, Busman M, Van der Lee T et al (2013) Birth, death and horizontal gene transfer of the fumonisin biosynthetic gene cluster during the evolutionary diversification of Fusarium. Mol Microbiol 90:290–306

Saikia S, Nicholson MJ, Young C, Parker EJ, Scott B (2008) The genetic basis for indole-diterpene chemical diversity in filamentous fungi. Mycol Res 112:184–199

Slot JC, Rokas A (2011) Horizontal transfer of a large and highly toxic secondary metabolic gene cluster between fungi. Curr Biol 21:134–139

Susca A, Proctor RH, Butchko RA, Haidukowski M, Stea G, Logrieco A, Moretti A (2014) Variation in the fumonisin biosynthetic gene cluster in fumonisin-producing and nonproducing black aspergilli. Fungal Genet Biol 73:39–52

Tokai T, Fujimura M, Inoue H, Aoki T, Ohta K, Shibata T, Yamaguchi I, Kimura M (2005) Concordant evolution of trichothecene 3-*O*-acetyltransferase and rDNA species phylogeny of trichothecene-producing and nonproducing fusaria and other ascomycetous fungi. Microbiology 151:509–519

Vaamonde G, Patriarca A, Pinto VF, Comerio R, Degrossi C (2003) Variability of aflatoxin and cyclopiazonic acid production by *Aspergillus* section *Flavi* from different substrates in Argentina. Int J Food Microbiol 88:79–84

Van Hove F, Waalwijk C, Logrieco A, Munaut F, Moretti A (2011) *Gibberella musae* (*Fusarium musae*) sp. nov.: a new species from banana is sister to *F. verticillioides*. Mycologia 103:570–585

Varga J, Kocsubé S, Suri S, Szigeti G, Szekeres A, Varga M, Tóth B, Bartók T (2010) Fumonisin contamination and fumonisin producing black Aspergilli in dried vine fruits of different origin. Int J Food Microbiol 143:143–149

Varga J, Frisvad JC, Kocsubé S, Brankovics B, Tóth B et al (2011) New and revisited species in Aspergillus section Nigri. Stud Mycol 69:1–17

Waalwijk C, Van Der Lee T, De Vries I, Hesselink T, Arts J, Kema GHJ (2004) Synteny in toxigenic Fusarium species: the fumonisin gene cluster and the mating type region as examples. Eur J Plant Pathol 110:533–544

Walton JD (2000) Horizontal gene transfer and the evolution of secondary metabolite gene clusters in fungi: an hypothesis. Fungal Genet Biol 30:167–172

Ward TJ, Bielawski JP, Kistler HC, Sullivan E, O'Donnell K (2002) Ancestral polymorphism and adaptive evolution in the trichothecene mycotoxin gene cluster of phytopathogenic Fusarium. Proc Natl Acad Sci USA 99:9278–9283. doi:10.1073/pnas.142307199

Wiemann P, Sieber CMK, von Bargen KW, Studt L, Niehaus EM, Espino JJ, Huß K, Michielse CB, Albermann S, Wagner D, Bergner SV, Connolly LR, Fischer A, Reuter G, Kleigrewe K, Bald T, Wingfield BD, Ophir R, Freeman S, Hippler M, Smith KM, Brown DW, Proctor RH, Münsterkötter M, Freitag M, Humpf H-U, Güldener U, Tudzynski B (2013) Deciphering the cryptic genome: genome-wide analyses of the rice pathogen Fusarium fujikuroi reveal complex regulation of secondary metabolism and novel metabolites. PLoS Pathog 9:1–35

Wong S, Wolfe KH (2005) Birth of a metabolic gene cluster in yeast by adaptive gene relocation. Nat Genet 37:777–782

Yu J, Chang PK, Ehrlich KC, Cary JW, Bhatnagar D, Cleveland TE, Payne GA, Linz JE, Woloshuk CP, Bennett JW (2004) Clustered pathway genes in aflatoxin biosynthesis. Appl Environ Microbiol 70:1253–1262

Chapter 10
How to Disentangle Changes in Microbial Function from Changes in Microbial Community

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Abstract Understanding the relations between microbial community structure, local edaphoclimatic conditions, and soil functions is fundamental to the progress of soil ecology. We identified soils with distinct textures and investigated the contribution of soil structure and microbial community to two soil functions performed by two nitrogen (N) cycling microbial functional groups. Part of the soil cores collected were sterilized in order to have the influence of the soil structure; the other part was used to obtain the soil microbial community associated with each of the four soil types studied. Soil textures and microbial communities were combined in all the possible combinations and allowed to rest for 15 days. After that, three dynamic soil characteristics were determined: potential nitrification, potential denitrification, and soil respiratory coefficient. At the same time, the number of copies of nitrification (amoA) and denitrification (nir and noz) functional genes was determined by real-time PCR.

Results showed that nitrification is much more dependent on the microbial community than denitrification, which is much related with soil chemical and physical properties, suggesting that the ability to predict microbial responses to environmental change can improve by understanding the context dependence of microbial structure–function relationships.

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10.1 Introduction

Understanding the relationship among community structure, ecosystem function, and environmental conditions is fundamental to the study of soil microbial ecology (Peralta et al. 2010).

Information on microbial communities is rarely explicitly considered in large-scale ecosystem models. However, recent work supports predictive relationships between microbial traits and ecosystem function (Allison and Martiny 2008). Many process-based models implicitly consider microorganisms by accounting for variation in factors that regulate microbial community composition, such as pH (Nicol et al. 2008), moisture (Nemergut et al. 2013), substrate availability, temperature (Shade et al. 2012), and salinity (Lozupone and Knight 2007). Yet, communities are not entirely determined by abiotic variables (Graham et al. 2016). The degree to which such factors affect the composition, functional traits, and activity of a given microbial community will affect the value of microbial data in predicting ecosystem processes beyond that of environmental factors alone. So the question is to understand if the composition of the soil microbial community is determinant to soil function. Graham et al. (2016) analyzing 82 datasets collected from an international team of collaborators suggested that greater understanding of microbial communities informed by ecological principles may enhance our ability to predict ecosystem process rates relative to assessments based on environmental variables and microbial physiology. Within the full dataset, environmental variables alone had greater explanatory power than either metrics of microbial community structure or microbial biomass. But taking in consideration that soil microbial processes require distinct abiotic conditions and most of these are determined by soil physical and chemical parameters, it is possible that the relation between soil structure and soil function is dependent on the soil function that is considered, even when considering functions that are performed by very specific functional groups.

We tried to develop a method to evaluate the relative importance of soil structural properties like texture and the soil microbial community on two soil functions performed by two "narrow" functional types: nitrification and denitrification.

10.2 Materials and Methods

10.2.1 Site Description

This study was conducted with soils from the Mediterranean basin differing mainly in their texture. All the soils were collected in a range of 5 km, in an area with typical Mediterranean climate, 450 mm of annual precipitation, with dry summers and moderately cold winters. August was the hottest month with an average

temperature of 27 °C and December the coolest month with an average temperature of 10 °C. Soils were collected in a degraded area, used for agriculture until 2007.

10.2.2 Sample Collection

Four transects, 100 m apart, were laid out parallel to the observed gradient in soil texture. Four areas with distinct soil textures were identified (areas A–D). In each area, six pooled soil samples were collected with a core (11 cm^2 area × 10 cm depth). No significant differences were observed for topography and plant composition.

Soil samples were collected in October 2014 to assess the relative effect of microbial community and soil physical and chemical characteristics on soil functions. Samples were transported on ice and stored at 4 °C prior to processing in the laboratory. A subsample was collected for storage at −80 °C for functional gene analyses.

10.2.3 Soil Chemical Analyses

Field moist soil was dried at 70 °C until constant weight, and moisture content was calculated from the proportion of water (by weight) to oven-dried soil. From each sample, a subsample of air-dried soil was ground into a fine powder and analyzed to determine total C and total N. Elemental analyses of C and N were completed using combustion methods (ECS 4010, COSTECH Analytical Instruments, Valencia). Soil pH was determined for a 5 g sample analyzed using a 1:1 soil:water ratio. In addition, ~5 g of field moist soil was extracted with 2 M KCl, and available ammonium (NH_4^+) and nitrate (NO_3^-) were analyzed in the extracts based on colorimetric analyses using an autoanalyzer (Dias et al. 2011). Texture analysis was performed on a composite 50 g sample from each plot using the hydrometer method (Gee and Bauder 1979). Two hundred (200) g of each soil sample were suspended in water (1:10 w/v), stirred at 150 rpm for 2 h at room temperature, and the extract sequentially filtered through 2 mm, 63 μm, 10 μm, and 2 μm mash. The fraction retained in the 2 μm filter was resuspended in 100 mL and used as a microbial soil suspension (Gee and Bauder 1979).

Intact soil cores were sterilized at 120 °C at 1 atmosphere for 2 h, allowed to cool down for 24 h, and the sterilization repeated for 3 times. Soil samples of 200 g were corrected for their water content (30–40 % of the WPC) and inoculated with a microbial community extracted from 200 g soil samples (from the same or from another soil) in order to have all the possible combinations between soil structure and microbial community. Inoculated soils were incubated under controlled conditions (14/19 °C) and 30–40 % WPC for 15 days. After the incubation period, soil

samples were taken to obtain soil potential nitrification and denitrification rates and microbial respiratory coefficient.

10.2.4 Potential Nitrification Assay

Potential soil nitrification rates were assessed using the short-term incubation. Approximately, 5 g aliquots of soil were weighed out into three replicate 125-mL flasks. Another 5 g of soil was weighed into one control bottle. Prior to incubation, 20 mL of 1 mM $(NH_4)_2SO_4$ and 0.1 mL of 2 M $NaClO_3$ were added to prepared soil samples. The replicates were shaken for approximately 5 h at about 130 rpm. The filtrate was collected from soils after the 5 h incubation and from control samples stored at −20 °C. This filtrate was analyzed for NO_2^--N based on a colorimetric assay. Calibration standards contained 0, 0.2, 0.4, 0.8, 1.0g NO_2^--N mL^{-1} (Kandeler 1996). Potential nitrification rate was calculated as the difference between control and incubated soil per mass of soil.

10.2.5 Potential Denitrification Assay

Potential soil denitrification rates were estimated using the acetylene inhibition method (Royer et al. 2004). In 125 mL Wheaton bottles, 90 mL of a 2 mM KNO_3 solution and 1.3 mL of chloramphenicol (100 mg mL^{-1}) and about 25 g of soil were combined. Bottles were sealed with septa-centered caps, shaken, purged with He for 5 min, and vented prior to beginning the assay. Prior to gas sampling, each bottle was shaken 5 min prior to sampling headspace to equilibrate N_2O in aqueous and sediment phases. Gas samples (15 mL) were collected at 0, 1, 2, and 3 h.

These gas samples were analyzed for N_2O using a Shimadzu 2014 greenhouse gas analyzer. Gas standards ranging from 0.1 ppm-v to 7.46 ppm-v N_2O were generated from 99 % N_2O (Grace Divisions, Deerfield, IL, USA). The N_2O concentrations of each sample per dry mass were plotted against time, and the slope of this line was the potential denitrification rate (ng N_2O g^{-1} dry mass h^{-1}). During the assay, N_2O production from each sample was linear for the majority of the samples measured. All gas samples were diluted prior to GC analysis in order for N_2O concentrations to remain within the range covered by the standard curve. Dry weight of soil samples was estimated based on gravimetric soil moisture determined for each sample.

10.2.6 DNA Extraction and Purification

Samples were freeze-dried prior to extraction of total genomic DNA using the FastDNA SPIN Kit for Soil (MP Biomedicals, Solon, OH). Genomic DNA was purified using acetyl trimethyl ammonium bromide (CTAB) extraction to remove contaminating humic acids (Sambrook and Russell 2001). DNA concentration was adjusted to a standard concentration of 20 ng L^{-1} prior to PCR and DNA-based microbial community analyses.

10.2.7 Denitrifier and Ammonia Oxidizer Gene Abundance

Quantitative PCR of nosZ and bacterial amoA genes were carried out in triplicate in 10 μL reactions. The nosZ gene was amplified using Nos1527F, 5′-CGC TGT TC (A/C/T) TCG ACA G(C/T)C A-3′ (Kloos et al. 2001) and nosZ1622R, 5′-CGC (G/A)A(C/G) GGC AA(G/C) AAG GT(G/C) CG-3′ (Throback et al. 2004). The bacterial amoA gene was amplified using PCR primers amoA-1F; 5-GGGG TTTCTACTGGTGGT-3′ and amoA-2R; 5-CCCCTCKGSAAAGCCTTCTTC-3′ (Rotthauwe et al. 1997). PCR reactions contained 1X SYBR green master mix (Applied Biosystems Inc., Foster City, CA), 0.4 μm of each primer, 0.5 μg μL^{-1} bovine serum albumin, and 2 μL of soil DNA of known concentration. Fragments were amplified with an initial denaturation step at 95 °C for 5 min, followed by 40 cycles of 95 °C for 1 min, 56 °C for 1 min, and 72 °C for 1 min. Standard curves were obtained based on serial dilutions of mixed PCR product from wetland soil samples. Reactions were analyzed on a 384-well Applied Biosystems 7900HT Fast Real-Time PCR System (Applied Biosystems Inc., Foster City, CA).

10.3 Results and Discussion

We observed that different combinations of soil factors influenced microbial structure and function between two nitrogen cycling functional groups: denitrifiers and ammonia oxidizers.

We used distinct soil types (Table 10.1) and extract their microbial components that were reinoculated into the four sterilized soil types in a way to obtain all the possible combinations between soil communities and structures (Table 10.2). Several parameters usually used to relate soil microbial community and soil function were used to assess the relative contribution of soil microbial community (inoculations) to nitrification and denitrification rates and/or respiratory coefficient in relation to non-dynamic soil properties.

Denitrifier and ammonia oxidizer community composition and abundance are known to vary in sensitivity to soil chemical and physical characteristics such as pH and organic carbon. In addition, the activities of these functional groups are quite

Table 10.1 Physical and chemical characteristics of the studied soils

	Soil			
	A	B	C	D
pH	5.6 ± 0.1	5.8 ± 0.3	5.8 ± 0.1	5.9 ± 0.1
WPC[a] %	30 ± 6	38 ± 5.0	45 ± 9	45 ± 7.5
C (mg^{-1} DW)	4.6 ± 0.7	6.7 ± 0.6	8.8 ± 1.2	10 ± 0.7
C/N	9.0 ± 0.9	10.1 ± 1.1	10.2 ± 0.8	10.3 ± 0.6
NO_2^- (ppm)	5.7 ± 0.2	7.7 ± 1.2	11.1 ± 1.1	43.0 ± 3.1
NH_4^+ (ppm)	10.0 ± 0.3	10.0 ± 0.7	11.0 ± 1.5	15.1 ± 1.1
Clay (%)	15.0 ± 1.2	18.8 ± 2.2	20.1 ± 2.5	25.2 ± 3.3
Lime (%)	26.8 ± 1.5	32.2 ± 5.5	44.1 ± 7.7	34.1 ± 5.8
Sand (%)	58.4 ± 5.2	49.0 ± 4.7	35.4 ± 5.5	30.8 ± 5.5

[a]Water content of the soil sample at the time of sampling
Results are the average of six determinations ± SD

Table 10.2 Results from the soil incubation with distinct soil microbial suspensions

		Soil structure			
Microbial community from soil		A	B	C	D
A	PNR	**8.1 ± 1.1**	6.8 ± 2.3	8.2 ± 1.1	6.3 ± 1.0
	PDR	**20 ± 10**	30 ± 5.0	50 ± 11	78 ± 8.1
	RQ	**1.5 ± 0.3**	1.5 ± 0.3	1.0 ± 0.1	0.7 ± 0.2
B	PNR	3.3 ± 1.7	**4 ± 3.2**	6 ± 1.0	5 ± 1.1
	PDR	26 ± 8	**32 ± 3**	58 ± 8	80 ± 10
	RQ	1.4 ± 0.1	**1.5 ± 0.2**	1.2 ± 0.3	0.7 ± 0.3
C	PNR	6.1 ± 0.3	5.3 ± 0.4	**5.6 ± 0.5**	6 ± 0.5
	PDR	48 ± 5	50 ± 6	**54 ± 4**	70 ± 9
	RQ	1.3 ± 0.1	1.2 ± 0.2	**1.2 ± 0.1**	0.7 ± 0.2
D	PNR	4.3 ± 0.4	4.5 ± 0.4	4.0 ± 0.4	**4.0 ± 0.1**
	PDR	50 ± 5.8	63 ± 6.1	65 ± 6.3	**72 ± 7**
	RQ	0.7 ± 0.2	0.6 ± 0.1	0.6 ± 0.2	**0.6 ± 0.2**

Potential nitrification rate (PNR) expressed as ng NO_2^- g DW^{-1} h^{-1}, potential denitrification rate (PDR) expressed as ng N_2O g DW^{-1} h^{-1}, and respiratory coefficient (RQ) expressed as mmol CO_2 $mmol^{-1}$ O_2. Results represent the mean of six repetitions ± SD. The results of the original microbial community in the respective soil structure are depicted in bold

responsive to oxygen (Francis et al. 2007; Kowalchuk and Stephen 2001; Wallenstein et al. 2006), and therefore, it is expected that it is affected by soil texture, as it was observed in the sterilized soils reinoculated with their original microbial communities (Tables 10.1 and 10.2). What is interesting from this dataset is that inoculating the bacterial community of a certain soil texture in a soil with less clay did not have much effect on the nitrification or denitrification rates, but its inoculation on a soil with more clay tended to increase the denitrification and decrease the nitrification rates (Table 10.2)

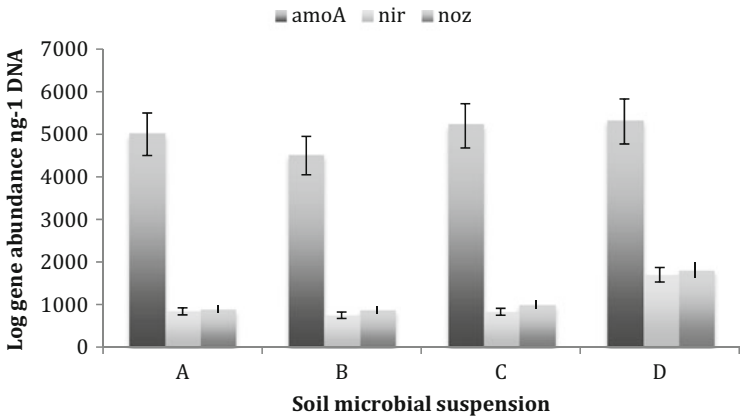

Fig. 10.1 Summary of *amoA* (*left*), *nirK* (*middle*), and *nosZ* (*right*) gene abundance based on quantitative PCR of the soil microbial suspensions isolated from the soils and used as inoculants. Bars represent the mean ($n = 3$) ± sd

In fact, soil texture is often correlated with chemical characteristics. Soil texture can influence how water and air travel through soil, thus influencing nutrient movement and surface area available for microorganisms (Brady and Weil 2002). In this study, soil factors that change over long timescales and are influenced by soil formation and management (soil texture, soil organic matter, pH) were very important to shaping denitrifier and ammonia oxidizer community structure, if evaluated by the functional gene abundance (Fig. 10.1).

Key functional genes for nitrification and denitrification were detected in all soil types. The total abundance of the denitrification genes *nosZ* and *nirK* were significantly higher in the microbial suspension of soil D, a clay soil ($P < 0.05$), which was consistent with the significantly higher denitrification potential observed in that soil (Table 10.2). Of the genes tested, *amoA* was the most abundant one. The gene *amoA* is largely associated with the bacterial phylum *Proteobacteria*, one of the largest divisions among prokaryotes, but its abundance did not differ among the tested microbial soil suspensions.

However, PNR were different across soils. Which may, at least at a first glance, imply that soil physical and chemical characteristics are more important to determine soil functions than the structure and function of soil microbial population (if analyzed on the basis of the abundance of the functional gene). Alternatively, we can argue that the physiological plasticity of the bacterial community that is present in the soil is also important, because it will determine the functionality of the gene (Strickland et al. 2009).

In order to assess if soil microbial community had potential to influence important soil functions such as nitrification and denitrification, we inoculated the distinct soils with microbial suspensions obtained from the other soils. And it was interesting to realize that the relation between soil function and microbial community was dependent on the function assessed (Table 10.2). Showing that the functional

response of microbial communities to their local environment is challenging to generalizations and varies according to the function of interest and microenvironmental conditions (Reed and Martiny 2007).

The distinct type of relation between soil structure and function is interesting because we are comparing functions that belong to the same biogeochemical cycle and that are performed by a "narrow" group of microorganisms. However, the inoculation of the distinct soil microbial suspensions affected the soil respiratory coefficient (the ratio between CO_2 evolution and O_2 consumption), which suggests the use of distinct carbon sources by the microbial community and therefore a broad interaction with the soil metabolic pathways. When using more recalcitrant carbon sources, the respiratory coefficient of the soil microbial community tends to decrease (Dilly et al. 2001).

Universal relationships between soil microbial diversity and function or services provided by the ecosystem have been proposed several times. But none seems suitable for explaining the majority of the relationships observed. The redundancy hypothesis assumes that species considered to be functionally redundant carry out the same function, and gain or loss in any one of these species does not influence overall function (Naeem et al. 2002; Petchey and Gaston 2006). In contrast, the rivet hypothesis assumes that there are key species that carry out or regulate a unique ecosystem function, and gain or loss of any one of these species directly affects function (Naeem and Wright 2003). However, demonstration of these generalizable structure–function relationships has been impossible due to the peculiar nature of the community response and because species within a community may present an assortment of responses to environmental variables (Cardinale et al. 2000; Chase 2010).

Our experiment demonstrates that in the short term the relation between structure and function is dependent on the function considered. But in order to distinguish environmental regulators in the short and long term, manipulative ecosystems experiments are desirable.

10.4 Future Perspectives

Recent decades have brought a surge in development in the knowledge of microbiomes, soil microbiomes in particular. However, we have to recognize that our knowledge of soil ecology is still very limited. We are currently considering the soil as a uniform identity, extracting the DNA and/or RNA, and analyzing the results in order to infer the phylogeny and functionality of the microbial community. Most of the time, no relation is found between the structure and function of the soil microbial communities. One of the reasons for this is that in most cases it is the rare species (representing <1 % of the soil microbial community) that determine the main soil functions. Another may be the fact that at the moment no major attention is given to soil spatial and temporal heterogeneity or microbial physiology, which

may be responsible for the fine-tuning between soil structure (physical and biological) and functionalities.

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References

Allison SD, Martiny JB (2008) Resistance, resilience, and redundancy in microbial communities. Proc Nat Acad Sci 105:11512–11519

Brady NC, Weil RR (2002) The nature and properties of soils, 13th edn. Prentice Hall, Upper Saddle Creek, NJ

Cardinale BJ, Nelson K, Palmer MA (2000) Linking species diversity to the functioning of ecosystems: on the importance of environmental context. Oikos 91:175–183

Chase JM (2010) Stochastic community assembly causes higher biodiversity in more productive environments. Science 328:1388–1391

Dias T, Malveiro S, Martins-Loução MA, Sheppard L, Cruz C (2011) Linking N-driven biodiversity changes with soil N availability in a Mediterranean ecosystem. Plant Soil 341:125–136

Dilly O, Bartsch S, Rosenbrock P, Buscot F, Munch JC (2001) Shifts in physiological capabilities of the microbiota during the decomposition of leaf litter in a black alder (*Alnus glutinosa* (Gaertn.) L.) forest. Soil Biol Biochem 33:921–930

Francis CA, Beman JM, Kuypers MMM (2007) New processes and players in the nitrogen cycle: the microbial ecology of anaerobic and archaeal ammonia oxidation. ISME J 1:19–27

Gee GW, Bauder JW (1979) Particle-size analysis by hydrometer—simplified method for routine textural analysis and a sensitivity test of measurement parameters. Soil Sci Soc Am J 43: 1004–1007

Graham EB, Knelman JE, Schindlbacher A, Siciliano S, Breulmann M, Yannarell A, Beman J, Abell G, Philippot L, Prosser J, Foulquier A, Yuste J, Glanville H, Jones D, Angel R, Salminen J, Newton RJ, Bürgmann H, Ingram LJ, Hamer U, Siljanen HM, Peltoniemi K, Potthast K, Bañeras L, Hartmann M, Banerjee S, Yu R, Nogaro G, Richter A, Koranda M, Castle S, Goberna M, Song B, Chatterjee A, Nunes OC, Lopes AR, Cao Y, Kaisermann A, Hallin S, Strickland MS, Garcia-pausas J, Barba J, Kang H, Isobe K, Papaspyrou S, Pastorelli R, Lagomarsino A, Lindström E, Basiliko N, Nemergut DR (2016) Microbes as engines of ecosystem function: when does community structure enhance predictions of ecosystem processes? Front Microbiol 7:214. doi:10.3389/fmicb.2016.00214

Kandeler E (1996) Potential nitrification. In: Schinner F, Öhlinger R, Kandeler E, Margesin R (eds) Methods in soil biology. Springer, Berlin, pp 146–148

Kloos K, Mergel A, Rosch C, Bothe H (2001) Denitrification within the genus *Azospirillum* and other associative bacteria. Aust J Plant Physiol 28:991–998

Kowalchuk GA, Stephen JR (2001) Ammonia-oxidizing bacteria: a model for molecular microbial ecology. Annu Rev Microbiol 55:485–529

Lozupone CA, Knight R (2007) Global patterns in bacterial diversity. Proc Nat Acad Sci 104: 11436–11440

Naeem S, Wright JP (2003) Disentangling biodiversity effects on ecosystem functioning: deriving solutions to a seemingly insurmountable problem. Ecol Lett 6:567–579

Naeem S, Loreau M, Inchausti P (2002) Biodiversity and ecosystem functioning: the emergence of a synthetic ecological framework. In: Loreau M, Naeem S, Inchausti P (eds) Biodiversity and ecosystem functioning: synthesis and perspectives. Oxform University Press, Oxford, pp 3–11

Nemergut DR, Schmidt SK, Fukami T, O'Neill SP, Bilinski TM, Stanish LF, Knelman JE, Darcy JL, Lynch RC, Wickey P (2013) Patterns and processes of microbial community assembly. Microbiol Mol Biol Rev 77:342–356

Nicol GW, Leininger S, Schleper C, Prosser JI (2008) The influence of soil pH on the diversity, abundance and transcriptional activity of ammonia oxidizing archaea and bacteria. Environ Microbiol 10:2966–2978

Peralta AL, Matthews JW, Kent AD (2010) Microbial community structure and denitrification in a wetland mitigation bank. Appl Environ Microbiol 76:4207–4215

Petchey OL, Gaston KJ (2006) Functional diversity: back to basics and looking forward. Ecol Lett 9:741–758

Reed HE, Martiny JBH (2007) Testing the functional significance of microbial composition in natural communities. FEMS Microbiol Ecol 62:161–170

Rotthauwe JH, Witzel KP, Liesack W (1997) The ammonia monooxygenase structural gene amoA as a functional marker: molecular fine-scale analysis of natural ammonia-oxidizing populations. Appl Environ Microbiol 63:4704–4712

Royer TV, Tank JL, David MB (2004) Transport and fate of nitrate in headwater agricultural streams in Illinois. J Environ Qual 33:1296–1304

Sambrook J, Russell D (2001) Molecular cloning: a laboratory manual. Dilly Soil Biol Biochem 33:117–127

Shade A, Peter H, Allison SD, Baho DL, Berga M et al (2012) Fundamentals of microbial community resistance and resilience. FMICB 3:417. doi:10.3389/fmicb.2012.00417

Strickland MS, Lauber C, Fierer N, Bradford MA (2009) Testing the functional significance of microbial community composition. Ecology 90:441–451

Throback IN, Enwall K, Jarvis A, Hallin S (2004) Reassessing PCR primers targeting *nirS*, *nirK* and *nosZ* genes for community surveys of denitrifying bacteria with DGGE. FEMS Microbiol Ecol 49:401–417

Wallenstein MD, Myrold DD, Firestone M, Voytek M (2006) Environmental controls on denitrifying communities and denitrification rates: insights from molecular methods. Ecol Appl 16: 2143–2152

Chapter 11
Legume-*Rhizobium* Symbioses: Significance for Sustainable Plant Production

Alexander P. Hansen

Abstract Biological N_2 fixation plays a crucial role for life on the planet. The most relevant terrestrial biological system for executing the fixation of atmospheric nitrogen is the legume-*Rhizobium* symbiosis. With the advent of the Haber-Bosch process, industrial N_2 fixation has reached parity with biological N_2 fixation during the last 100 years, allowing an increase in agricultural production to feed currently more than seven billion inhabitants on the planet. But this does not come without environmental costs. While the Haber-Bosch facilitates a maximization of agricultural production, biological N_2 fixation is the preferred choice for sustainable agriculture. This chapter provides an insight in the legume-*Rhizobium* symbiosis and puts it in perspective with regard to its potential and limitations for sustainable plant production.

11.1 Introduction

Biological N_2 fixation evolved already 4000–2500 million years ago in early Archean oceans (Towe 2002), probably in response to reductions in the amounts of nitrogen fixed *via* lightning discharges or releases from volcanic eruptions. Beneficial effects of N_2 fixation are known to humans for at least 2000 years, since the Romans practiced already cropping systems combining legumes and non-legumes, due to their observation that the non-legumes benefitted from such mixed cultivation. Devoid of any explanation for this effect, elucidation arrived in the nineteenth century. Without conclusive prove, Boussingault (1838) suggested that legumes were fixing nitrogen from the air. Although Justus von Liebig, the leading organic chemist at the time, contradicted this claim, subsequent experiments of two of his German compatriots, Hellriegel and Wilfarth (1888), provided conclusive prove of nitrogen fixation of nodulated peas.

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About at the same time, development of a technical processes to fix atmospheric nitrogen progressed further, leading eventually to the commencement of industrial nitrogen fixation in the small German town of Oppau in 1913. Although the original intention for the industrial ammonia production, utilizing the process developed by Fritz Haber and Carl Bosch, was to provide Germany with a source of ammonia for the production of explosives used in WWI, thus compensating the allied trade embargo on Chilean saltpeter, the process later permitted for the first time large-scale nitrogenous fertilizer production. This formed the basis for the "Green Revolution," which started in the 1940s in Mexico with the work of Norman E. Borlaug on the development of disease-resistant, high-yielding wheat varieties. He is generally perceived as the "father" of the "Green Revolution" and received for his work the Nobel Prize in 1970. Production of improved varieties of crop plants in combination with the availability of industrial nitrogen fertilizer was the basis for the enormous increase of agricultural production, which ensured the survival of millions of people and allows more than seven billion people to inhabit the planet today. It has recently been estimated that now approximately half of the nitrogen received by the world's crops (Smil 2011) and protein in humans consists of nitrogen, originally fixed by the Haber-Bosch process. Two decades earlier, estimates still attributed only one third of global nitrogen fixation to industrial production (Burris and Roberts 1993). Although solid data for such estimates are difficult to obtain and consequently subject to errors, it nevertheless indicates, in line with many other sources (FAO 2012), that industrially fixed nitrogen is increasing its contribution to crop production. The rest of the fixed nitrogen comes from biological nitrogen fixation (mainly leguminous corps), organic recycling, and atmospheric deposition. Hence, without the Haber-Bosch process, less than half of the world's population could be sustained today.

Since this process

$$N_2 + 3H_2 \rightarrow 2NH_3 \quad (\Delta H = -45.8 kJ \cdot mol^{-1})$$

requires high pressure (200 atmospheres) and high temperature (400 °C), it is very energy consuming. The required energy is generated primarily via fossil fuel. Consequently, today's agriculture is responsible for a substantial carbon footprint. The amount of CO_2 production, directly linked to industrial transformation of N_2 to NH_3, is estimated to be approximately 80 million metric tons or about 1 % of the global CO_2 emissions. Although 1 % of the global CO_2 emission is already a significant contribution to the global liberation of climate gas, a more significant detrimental effect on global climate of intense agriculture is the release of N_2O into the atmosphere. N_2O is liberated in connection with N fertilizer application in intensive agriculture and a very potent "greenhouse gas." According to Prather and Ehhalt (2001), the global warming potential (GWP) of N_2O over a 100-year average is 296 times larger than an equal mass of CO_2. This is due to the long residence time of N_2O in the atmosphere, exceeding 120 years. Consequently, even the production of biofuels, such as biodiesel from rapeseed or bioethanol from maize, does not lead

to a reduction of "greenhouse gas" emissions, due to the additional liberation of N_2O, which overcompensates the savings on net CO_2 emission from reduced quantities of burned fossil fuel (Crutzen 2008). Due to the high activity of N_2O as a climate gas—in addition to its impact on the ozone layer Crutzen (1970)—highly efficient agriculture, aiming for maximal yields, releases N_2O in amounts, which are very significant for climate change. Estimates to quantify the liberation of N_2O are numerous, but include a substantial amount of uncertainty. While outlining the challenges related to global quantifications of N_2O emissions, Mosier et al. (1998) estimated the total annual amount of global N_2O emissions from agricultural soils, animal production and aquatic systems to be in the vicinity of 6.3 TgN_2O, which exceeds previous estimates significantly. If one takes the GWP of N_2O into account, the impact of industrial nitrogen fixation, its intensive agricultural usage and the resulting N_2O emissions, the relevance for global warming becomes obvious.

In addition excessive quantities of nitrogen are known to have detrimental effects as pollutants in the air, in water, and in the soil.

NO_x, i.e., nitric oxide (NO) and nitrogen dioxide (NO_2), are produced from the reaction among nitrogen, oxygen, and also hydrocarbons during combustion. NO_x gases are produced in motor vehicles and naturally during lightning. Due to high density of motor vehicles, it is one of the major pollutants in large cities and a key component in tropospheric ozone production in a reaction with carbon monoxide (CO) and volatile organic compounds (VOCs) in the presence of sunlight. While stratospheric ozone (the ozone layer) plays a very important role in the absorption of the sun's ultraviolet (UV) radiation, ground-level ozone has serious health effects by affecting the respiratory system of humans. Reduced lung function, respiratory infections, and asthma are common consequences of high concentrations of tropospheric ozone. In addition, if NO_x gets in contact with moisture in the atmosphere, it turns into nitric acid, which is the nitrogen component of acid rain (Singh and Agarwal 2008) and responsible for acidification of water bodies, the decrease of soil fertility, and ultimately the reduction of productivity of ecosystems and cropping systems.

Nitrate is highly soluble, rapidly leached out of the root zone of plants and enters the groundwater or rivers. Consequently, lakes or coastal zones become eutrophic, frequently leading to algal blooms and excessive growths of water hyacinths (*Eichhornia crassipes*) or other aquatic plants. If too much oxygen is removed, the water body develops a "dead zone" with disastrous effects on endogenous aquatic flora and fauna.

Low efficiency of nitrogenous fertilizer uptake of many crop plants, combined with excessive application of nitrogen, also leads eventually to soil saturation. Due to the fact that other nutrients are leached along with the nitrogen and negative effects on the carbon balance of the soil, it eventually reduces the soil productivity after an initial increase (Matson et al. 1999).

If one carefully considers the various positive and negative effects of the industrial production of fixed nitrogen, the question arises, if the elimination of one of the major restricting factors for agricultural production really allows the

increase of the long-term carrying capacity of the globe or whether it is more of a temporary effect. In other words, is the current intensive agricultural system really sustainable? To elucidate this question further, biological nitrogen fixation and its significance for sustainable plant production will be discussed here in view of environmental and social aspects.

11.2 Biological Systems for Nitrogen Fixation

As mentioned above, the fixation of the triple-bonded molecular nitrogen (N_2) requires a substantial amount of energy. This energy is provided in industrial fixation *via* fossil fuels, while diazotrophs (N_2-fixing organisms) rely on light or chemical energy to produce ammonia. All diazotrophs have a prokaryotic cell structure and produce ammonia at ambient temperature and atmospheric pressure. The capacity to fix nitrogen in these organisms relies on the nitrogenase enzyme system, which, at 16 ATPs hydrolyzed per N_2 fixed, carries out one of the metabolically most expensive processes in biology (Simpson and Burris 1984). The primary abiotic natural source of fixed nitrogen comes from lightning discharges and accounts globally for <100 Tg N ha^{-1} $year^{-1}$, while biologically fixed nitrogen by diazotrophs is estimated to be in excess of twice the quantity (Falkowski 1997).

Diazotrophs can be differentiated in three groups with regard to their strategy to fix nitrogen in terrestrial systems:

- Free living
- Associative
- Symbiotic

Irrespective of this general differentiation, one has to be aware of the fact that differences are not always without ambiguity and representatives of each category may subscribe to two groups depending on environmental parameters. One other major distinction between the diazotrophs is the form in which they acquire their energy. Some are carbon heterotrophic (e.g., *Azospirillum*), while others are carbon autotrophic, mostly relying on light energy (e.g., *Trichodesmium erythraeum*). Among free-living, carbon autotrophic diazotrophs, cyanobacteria are in terms of nitrogen fixation performance most active (>80 kg N ha^{-1} $year^{-1}$). Their habitat ranges from arctic regions to hot springs. They are found in soil and fresh- and salt water. Their agricultural significance is greatest in paddy fields used for the cultivation of rice (Ladha and Reddy 2000). Carbon heterotrophic free-living nitrogen-fixing bacteria (e.g., *Azospirillum*, *Clostridium*) often suffer from carbon limitation leading to lower nitrogen fixation performance (Ladha and Reddy 2000). Consequently, their contribution of fixed nitrogen to terrestrial systems is rather low (≤ 2 kg N ha^{-1} $year^{-1}$).

Conditions in the tropics are most favorable for free-living diazotrophs occupying the phyllosphere. Here desiccation is less frequent generating conditions, which allow the heterotrophic diazotrophs to utilize lost combined carbon from leaves as

an energy source for N_2 fixation. Fallen pollen grains, leaf leachates, and even the cuticle itself have been suggested as a carbon source for phyllospheric microbes (Sebastian et al. 1987). Among the numerous diazotrophs identified in the phyllosphere are genera like *Azotobacter*, *Pseudomonas*, or *Klebsiella*, which are normally found in soil. A study of Nandi and Sen (1981) indicated that under N-limited conditions, phyllospheric diazotrophs are capable to increase plant growth, similar to mineral leaf fertilizer. They could demonstrate that after 8 weeks of growth, plants inoculated with diazotrophs increased their nitrogen content by a factor 4 compared to control plants. Phyllospheric nitrogen-fixing organisms are attributed with an annual nitrogen fixation performance of 10–-20 kg N ha^{-1} $year^{-1}$.

Rhizospheric associations are considered more productive in nitrogen fixation than any of the phyllospheric variants. In such associations diazotrophs, colonizing intercellularly the inner tissues of plants without causing any apparent damage, are referred to as an endophyte (e.g., *Gluconacetobacter* sp. or *Azoarcus* sp.). *Azospirillum* sp. are facultative endophytic, thus being found both inside the root tissues and on the root surface (van Dommelen and Vanderleyden 2007). Since a considerable proportion of carbon fixed in photosynthesis is released into the rhizosphere as root exudates, this habitat provides diazotrophs with a rich source of energy for N_2 fixation. Although plants benefit from the fixed nitrogen mainly after the death of the bacteria, due to the dynamics of the system, a measurable benefit can be achieved with rates of nitrogen fixation reaching up to ca. 80 kg N ha^{-1} $year^{-1}$, depending on conditions.

Symbiotic nitrogen fixation is the most productive form of terrestrial biological nitrogen fixation. In these systems the plant benefits directly from the fixed N_2, while the endophyte encounters optimal conditions for survival. Such symbiotic systems are found in legumes as well as non-legumes. In non-legumes the actinomycete *Frankia* and the blue-green algae (cyanobacteria) *Nostoc* and *Anabaena* are of particular relevance. Cyanobacterial symbioses normally rely for the infection process of the endophyte on cavities in plants. Often the respective plant parts are specifically modified to accommodate the symbiont. Some plants (e.g., *Macrozamia* sp.) have specialized, apogeotropic roots, which may occur from several centimeters blow the ground to above the soil surface. Due to their structure, these roots are generally referred to as coralloid roots and host the cyanobacteria (Bergman et al. 1992).

The *Azolla*/*Anabaena* system is the most important cyanobacterial symbiosis in agriculture and utilized widely in rice cultivation. The genus *Azolla* comprises aquatic ferns, widely distributed throughout the world. *Anabaena* enters developing leaves of the fern on the dorsal side, where a cavity exists in young leaves. The cavity contains mucilage and hairs, which function as transfer cells. In the early stage of the symbiosis, the hairs facilitate the carbon transfer to the endophyte. Newly produced hairs eventually allow the host to assimilate the ammonia produced by the increasing number of N_2-fixing cells (heterocysts) of *Anabaena* (Calvert et al. 1985)

The actinomycete *Frankia* is capable to form nodules with his host (e.g., *Alnus* sp., *Casuarina* sp., *Allocasuarina* sp.) to accommodate the symbiotic system. Root infections and subsequent nodule development are in many ways analogous to the process occurring in legumes (Tjepkema et al. 1986; Hansen 1994). This form of nitrogen-fixing symbiosis plays in agriculture no significant role.

Symbioses with legumes as the macrosymbiont and species of the genera *Rhizobium* or *Bradyrhizobium* as the microsymbiont are probably the most important symbiotic systems and best studied (Sprent and Sprent 1990; Long 1992; Somasegaran and Hoben 1994; Epping et al. 1994). Microsymbionts are largely host specific. Therefore, a recognition system between the symbiotic partners is required in order to allow the bacterial penetration and the subsequent formation of the nodules, i.e., the specific plant organ hosting the microsymbiont and the nitrogen fixation system. Early steps of nodule formation involve various forms of signal exchange, bacterial attachment, and penetration into the root cortex. Isoflavonoids, flavonoids, isoflavones, and highly specific lipo-oligosaccharides have been attributed with a signaling function in this context. Crack entry or more commonly root hair curling (Figs. 11.1 and 11.2) allows the formation of annual or perennial nodules, which contain large numbers of the microsymbiont.

The infection process is extensively discussed in the literature (e.g., Kijne 1992; Shrivastava et al. 2014) and will be described here only in a summarized form. Nodule formation can be arbitrarily divided into the stages of preinfection, infection, nodule formation, and differentiation. In the first step of infection, rhizobial cells attach to the root hair. Subsequently, a host-specific tight root hair curling is induced, followed by entrapment of the bacteria. This being a prerequisite for ingestion induces host cell wall degradation and invagination the host plasma membrane. The tubular invagination of the plasma membrane extends during the progress of the infection process, which is accompanied by a deposition of cell wall material in the subapical region of the invagination membrane. This structure has been described as the infection thread (see Fig. 11.2) and contains the invading rhizobia, which are eventually released into the host cells (infected cells) in a process resembling endocytosis. In conjunction with the initial infection thread development, growth of the tip of the root hair is terminated. Subsequently, the formation of a nodule primordium starts (Fig. 11.2) with cell division in the inner cortex at several cells ahead of the advancing infection thread. Neighboring cortical cells commence with mitotic activity leading to a meristematic area, eventually giving rise to a differentiated nodule (Kijne 1992). The "infected cells" allow the bacteria to differentiate into bacteroids. Growth-inhibiting substances released by the host have been implicated to serve as a controlling factor during this stage (Ozawa and Tsuji 1993). In morphological and physiological terms, bacteroids are modified bacteria, which, during the differentiation process, loose their cell wall and become substantially enlarged and enclosed by a peribacteroid membrane. Biogenesis of the enclosing membrane is thought to involve the plant plasmalemma and cell membrane systems as well as bacterial cells. Each peribacteroid membrane may encircle one or numerous (up to around 20) bacteroids depending on the plant

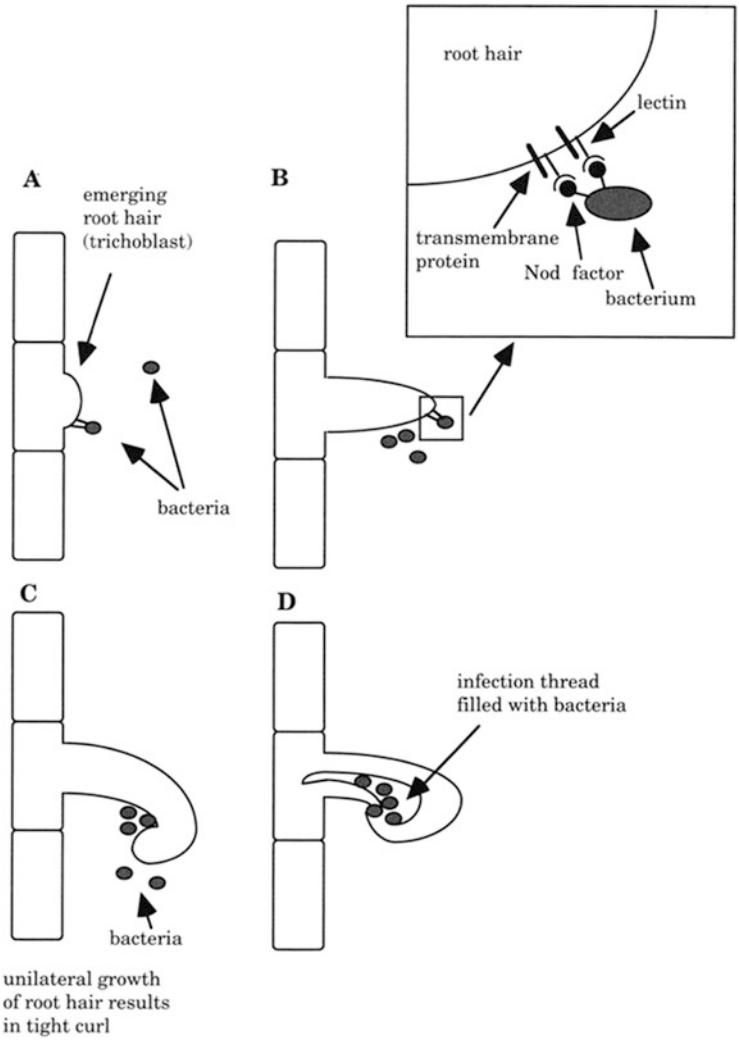


Fig. 11.1 Schematic drawing of early steps of rhizobial attachment (**a**), trichoblast extension (**b**), induction of root hair curling (**c**), and (**d**) penetration of rhizobia with infection thread formation (Hansen 1994). Detailed drawing in expanded box indicates details of the rhizobial attachment following the Nod factor reception model of Hirsch (1992)

species and age of the nodule. The region of fully differentiated, infected cells is the nitrogen-fixing zone of the nodule (see Fig. 11.2).

One can differentiate between determinate and indeterminate nodules, forming the two major types of symbiotic organs in legumes. The nodule morphology is determined by the macrosymbiont rather than the rhizobial strain. Consequently, each plant species characteristically forms only one type of nodules. However, according to a study of Iqbal and Mahmood (1992), *Leucaena leucocephala* forms

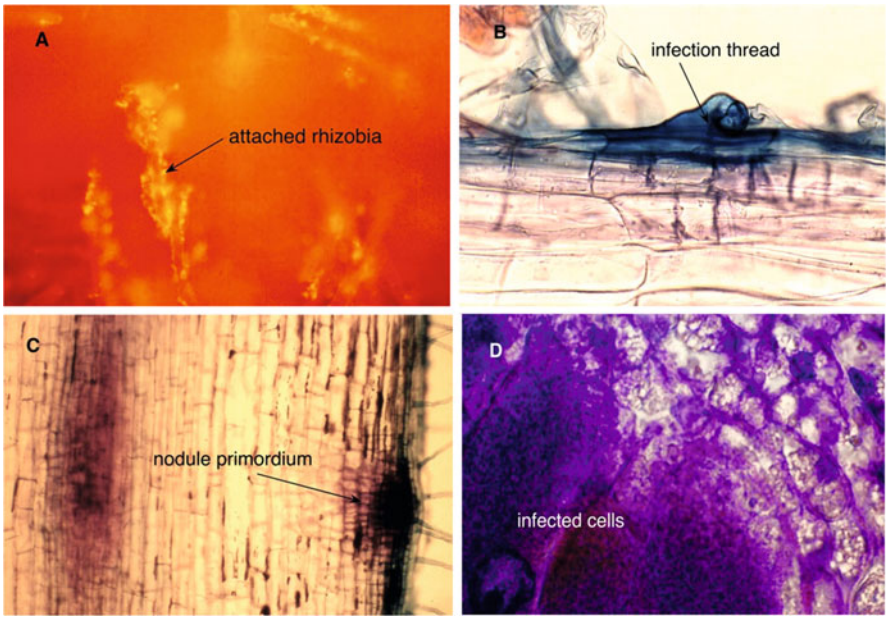


Fig. 11.2 Rhizobial attachment on *Glycine max* (**a**), curled root hair of *Phaseolus vulgaris* with infection thread (**b**), nodule primordium in *Phaseolus vulgaris* (**c**), and *dark purple* infected cells on the *left side* and *lighter-colored* uninfected cells on the *right side* in *Glycine max* (**d**). From Hansen (unpublished)

an exception, being capable to produce determinate and indeterminate nodules. Although symbiotically active nodules are typically found on roots, stem nodulation is also known in some genera, e.g., *Sesbania* or *Aeschynomene* (Fig. 11.3). Stem nodulation appears to be influenced by environmental conditions, e.g., paddy soils *versus* upland location (Joshua et al. 1992).

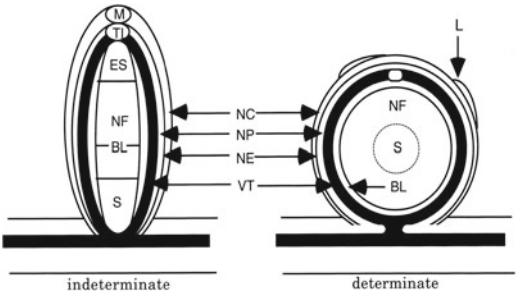
The main difference between determinate and indeterminate nodules is the extent of the lifespan of their meristems (Figs. 11.4 and 11.5), being persistent in the latter, but not in the former case. Among the commonalities of the two, one should mention that they have similar central and peripheral tissues. While the central tissues house the endophyte in the infected cells, the interstitial cells in the same region remain uninfected. The nodule cortex, the endodermis, and nodule parenchyma form part of the peripheral tissue and contain the vascular bundles. Cell division activity in determinate nodules is only transient, leading to nodules of a more or less spherical shape, typically found on soybean (*Glycine max*), mung bean (*Vigna radiata*), and many other crop plants. Indeterminate nodules possess a persistent meristem, producing initials that remain part of the meristem and further give rise to differentiated cells, which in the process become infected. Due to the continued meristematic activity cylindrical, bifurcated or coralloid nodules of perennial nature are formed (Fig. 11.5).

Fig. 11.3 Pictures of root and stem nodules of *Sesbania rostrata* (from Hansen, unpublished)

11.3 Metabolism of Biological Nitrogen Fixation

The nitrogenase enzyme complex is responsible for the nitrogen fixation process in all eubacteria (Hinnemann and Nørskov 2006). The process requires 16 mol ATP for reduction of molecular nitrogen to 2 mol NH_3. Depending on physiological conditions and the type of nitrogenase, more than 50 % of the energy may be used to produce molecular hydrogen. Thus, the energy demand can drastically vary. The enzyme shows high sensitivity to free oxygen. Consequently, an efficient carbon metabolism has to be maintained in the nodule to ensure O_2-limited conditions of the symbiotic system (Lance and Rustin 1984; Vance and Heichel 1991; Werner 1992).

Three forms of the nitrogenase enzyme complex are currently known. The most common and earliest discovered one is the molybdenum nitrogenase, containing two metalloproteins, the iron (Fe) protein and the molybdenum-iron (MoFe) protein. Vanadium nitrogenases are the second form of the N_2-fixing enzyme, considered to be a backup system, since it is encoded along with the molybdenum analog



Site of initial cell division	Inner cortex	Outer cortex
Nodule growth	Persistent	After initial establishment, mainly cell expansion
Infection thread	Broad	Narrow
Export products	Amides	Ureides
Site of N_2 assimilation	Infected cells	Interstitial cells
Life span	Perennial	Annual

M, meristem; TI, thread invasion zone and zone of plan cell expansion; ES, early symbiotic zone with symbiosomes; NF, zone of nitrogen fixation; S, senescent zone; NC, nodule cortex; NE, nodule endodermis; NP, nodule parenchyma, VT, vascular tissue; BL, boundary layer; L, lenticel in peride

Fig. 11.4 Major differences between indeterminate and determinate nodules [from Hansen (1994)]

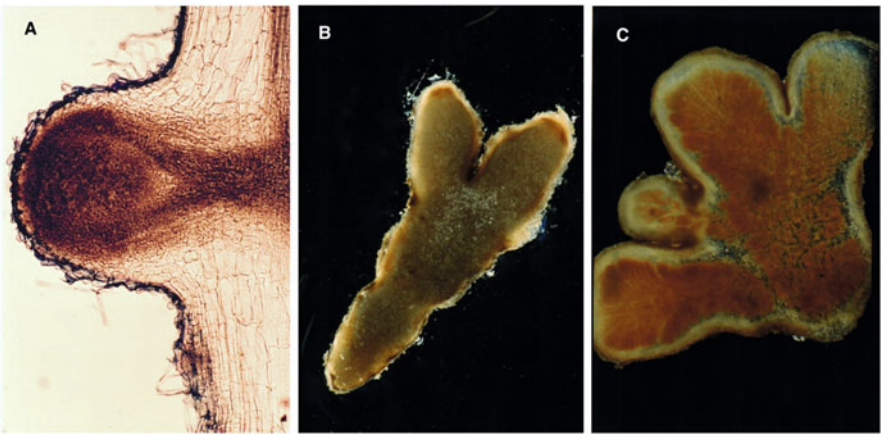


Fig. 11.5 Determinate root nodules of *Phaseolus vulgaris* (**a**); indeterminate, bifurcated nodule of *Acacia extensa* (**b**); and indeterminate, coralloid root nodule of *Mimosa pudica* (**c**) from Hansen (unpublished)

and expressed in case of Mo deficiency. Vanadium nitrogenases are also more efficient and are predominately expressed at low temperatures. The third form of nitrogenase is an iron-only variety, expressed under conditions of Mo and V deficiency. Vanadium nitrogenases have been isolated form *Azotobacter vinelandii* and *Azotobacter croococcum*, while Fe-only nitrogenases were found in *Rhodobacter capsulatus*, *Rhodospirillum rubrum*, as well as *Azotobacter vinelandii* (Krahn et al. 2002). The non-molybdenum nitrogenases may have a technological application due to much higher rates of hydrogen evolution. As mentioned above, in vanadium and Fe-only nitrogenases, 50 % or more of the energy is allocated to this process—twice as much as in molybdenum nitrogenases (Rehder 2000). Other technical applications may be possible for vanadium nitrogenases, due to their ability to reduce carbon monoxide (CO) to ethylene (C_2H_4), ethane (C_2H_6), and propane (C_3H_8) (Lee et al. 2010).

The reduction of N_2 by the most common molybdenum nitrogenase has been outlined in numerous publications (e.g., Newton 1992; Orme-Johnson 1992; Kim and Rees 1992a, b) and can be summarized in three major steps:

1. Reduction of the Fe-protein by electron carriers (e.g., flavodoxin)
2. Transfer of the electrons to the MoFe-protein in a MgATP-dependent process
3. Electron and proton transfer to the substrate

The reactions and components, which are part of the nitrogen fixation process, are depicted in a simplified schematic drawing in Fig. 11.6. For review and more details regarding the symbiotically active compartments and their function, see Werner (1992) or Hinnemann and Nørskov (2006).

11.4 Measurement of Nitrogen Fixation

Measurement of biological nitrogen fixation is essential for the determination of global inputs of symbiotically produced combined nitrogen. Due to the magnitude of biological nitrogen fixation (see above), the understanding and management of the nitrogen cycle on Earth is highly dependent on accurate data. However, precisely the question of accuracy has been discussed in the scientific literature *in extenso*, since all known methods are subject to significant drawbacks leading to potentially large errors. Consequently, most studies are retreating to the usage of the term "estimate" rather than "measurement," despite the fact that certain parameters can be measured. The translation of such measurements to quantities of N_2 fixed on a larger scale is a problem often approached, but never completely resolved—so far. Estimates can be improved if the technique of choice is combined with other methods to confirm results. In principle one can differentiate two groups of techniques: direct and indirect methods. Direct methods use nitrogen itself to determine N_2 fixation. The oldest direct approach is the nitrogen-difference technique, which is based on the assessment of the difference between the amount of N in a legume crop compared to a non-N_2-fixing control crop. A variant of that approach is the

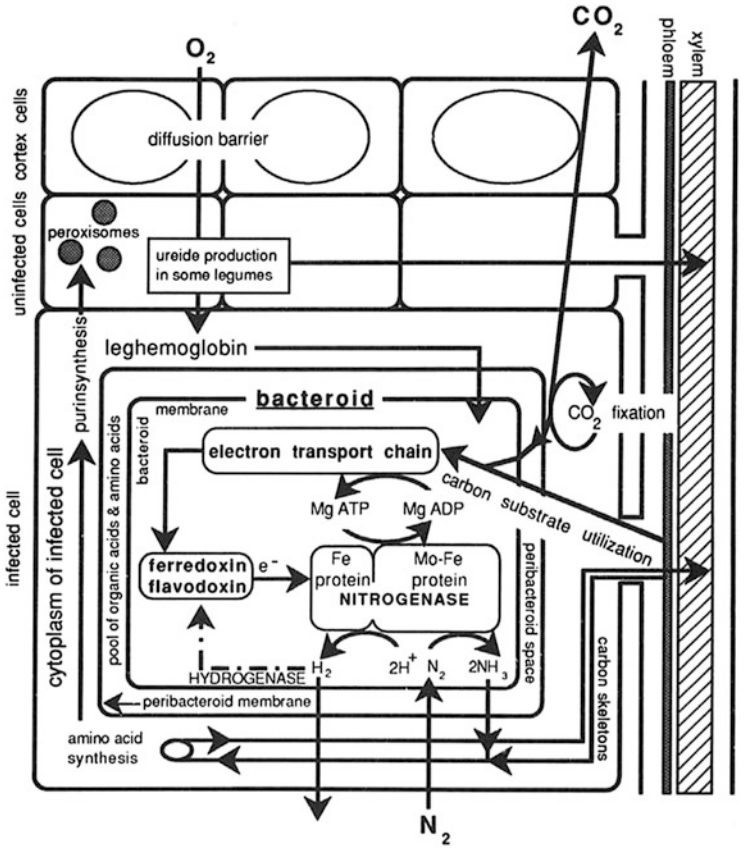


Fig. 11.6 Scheme of the components and reactions during N_2 fixation by the molybdenum nitrogenase enzyme complex in a bacteroid and adjacent cells. The Fe-protein reduces the MoFe-protein and becomes itself reduced *via* the electron surplus of flavodoxin or ferredoxin. Alternatively, electrons pass from NAD(P)H to flavodoxin *via* proteins or to the electron transport chain. Hydrogenase is responsible for the recycling of H_2 produced during N_2 fixation

N-balance method, which is based on the principle that the plant/soil system will accumulate nitrogen over the years, if there is an input form N_2 fixation. However, due to N losses from denitrification, volatilization and leaching, accuracy is limited. In addition inputs from pollution-related N compounds or lightning may restrict the accuracy of the method further, unless inputs and outputs can be assessed (Giller and Merckx 2003). Other direct methods rely on the stable isotope ^{15}N. Indirect methods are reliant on alternative substances indicating N_2 fixation, namely, these are the acetylene reduction method and the xylem-solute technique.

11.4.1 The N Difference Method

The N difference method compares the N accumulation of N_2-fixing plants to adjacent non-N_2-fixing plants. This comparison assumes that N_2-fixing plants assimilate the same amount of soil mineral N as the adjacent nonsymbiotic plants. Due to morphological and physiological differences between the compared species, the method is rather inaccurate unless the soils are N-limited, and the symbiotic activity of the legumes is high (Peoples and Herridge 1990). Consequently, this method has been mainly used prior to the refinement of other approaches.

11.4.2 Stable Isotope Techniques

The stable nitrogen isotope ^{15}N has been used to measure nitrogen fixation for more than 60 years. However, only the wider availability of mass spectrometers for sample analysis allowed a more common usage of this labeled form of nitrogen. In laboratory settings the isotope can be supplied as $^{15}N_2$, which allows the measurement of the $^{15}N_2$ fixed in the symbiotic system in plants. Alternatively, the soil N pool can be enriched with ^{15}N permitting the measurement of nitrogen fixation by calculating the dilution of the soil nitrogen from the fixation of atmospheric nitrogen ($^{14}N_2$). A variant of this technique is based on the preferred utilization of lighter isotopes of elements by organisms (for review see Bowen 1960). This preference applies to nitrogen as well, leading to a natural enrichment of soil nitrogen with ^{15}N, avoiding the requirement to add ^{15}N-enriched materials. In recent years the utilization of this technique has increased, due to greater accessibility of high-precision isotope-ratio mass spectrometers. Despite some limitation of this method, it has been widely used in agricultural systems, while in natural systems, its application is more limited due to increased methodological complications (Hansen and Pate 1987b; Hansen 1994; Boddey et al. 2000; Gehring and Vlek 2004).

11.4.3 Xylem-Solute Technique

The xylem-solute technique offers another option to assess nitrogen fixation. In symbiotically active legumes, N compounds in the xylem originate from the soil as well as from assimilation products of N_2 fixation coming from the nodules. In cases where pronounced differences of xylem N-solute composition in dependence of the N source can be established, respective "indicator molecules" can be used to assess nitrogen fixation activity (Unkovich and Pate 2000; Peoples et al. 2002). However, such "indicator molecules" are not present in the xylem sap of all legumes. Roughly one can differentiate two classes of legumes with specific export products. While

most legumes with determinate nodules transport the majority of their fixation products in form of ureides (i.e., allantoin and allantoic acid), legumes with indeterminate and other form of nodules generally transport their fixed nitrogen predominantly as the amino acid asparagine and glutamine. Agriculturally important legumes—like soybean (*Glycine max*), black lentil (*Vigna mungo*), adzuki bean (*Vigna angularis*), and common bean (*Phaseolus vulgaris*)—export ureides as nitrogen fixation products from their nodules to the shoot (e.g., Hansen et al. 1995). In these species, the ratio of ureide N to total N in the xylem sap is closely correlated with the nitrogen fixation performance of the plants. Consequently, this method has been successfully applied in numerous studies with crop plants (Hardarson and Danso 1993; Hansen 1994).

11.4.4 Acetylene Reduction Assay

Another method reliant on "indicator molecules" is the acetylene reduction assay. The nitrogenase enzyme complex is not substrate specific and is capable of reducing acetylene (C_2H_2) to ethylene (C_2H_4), even with a higher affinity than it has to N_2. In fact, acetylene is already at a concentration of ≈6 % a competitive inhibitor of N_2 fixation (Schöllhorn and Burris 1967). Both gases can easily be quantified by using a gas chromatograph equipped with a flame ionization detector. Hence, the assay is a sensitive measure of nitrogenase activity at a specific point in time. However, as a competitive inhibitor of N_2 fixation, the activity measured by acetylene reduction may not precisely reflect the enzyme activity in the absence of acetylene. In addition measurements require the enclosure of the sample in a gas-tight container to evaluate ethylene production. Physical disturbance and substrate change are only some of the factors, which may lead to errors if results are extrapolated for field studies. Consequently, the initial hype in favor of the method ebbed down when a number of critical reports appeared (Minchin et al. 1983; Minchin et al. 1986; Hansen et al. 1987).

All the above methods have their flaws and strengths. A major problem is mostly the extrapolation from measurements on a number of individual plants. This applies to the acetylene reduction assay as well as the xylem-solute technique. Depending on the experimental setup, also ^{15}N-involving methods can subscribe to the same challenge. Hence, integrated methods, like the N difference method, have a clear advantage in that respect, since they provide an estimate over time and do not require extrapolations from individually measured plants (for more extensive comparison of the methods, see, e.g., Hansen (1994)). However, it is not a method of precise measurement, but rather a comparison between two different species and subject to all the problems associated with a comparison of plants, with different physiological and morphological characteristics. Hence, the term "estimate" is indeed more appropriate than "measurement." This is the reason why exact measurements of global inputs of biological nitrogen fixation are so difficult and

constantly are in need of correction (e.g., Delwiche 1970; Burris 1980; Galloway et al. 1995; Herridge et al. 2008).

11.5 Environmental Impact on Biological N_2 Fixation

Due to the high-energy demand of N_2 fixation and the dependence of the microsymbiont to get this energy from the macrosymbiont, anything affecting plant photosynthetic activity will also eventually influence nitrogen fixation. However, the symbiotic system is usually more sensitive to adverse conditions than the plant. Hence, reductions in symbiotic activity can normally be detected prior to effects on plant grows. In general, the impact of environmental conditions on nitrogen fixation is rather complex, involving aspects of population dynamics of rhizobia, infection, nodulation, host plant development, and so on. Many of the effects have been outlined in the literature (e.g., Wood and Cooper 1988a, b; Sprent and Sprent 1990). This chapter will cover some of the most important factors without a detailed outline of the various interactions between specific parameters.

11.5.1 Temperature

Temperature will affect nodulation and symbiotic activity. However, the effects may vary depending of rhizobial strain, the host plant, and their respective adaptations to specific temperatures. Not surprisingly low temperatures less affect plants and rhizobial strains from cold climatic regions than those from warmer climates. Despite such adaptations of different species, within one species, there is generally a fairly narrow range of adjustment to extreme temperature changes (Hungria et al. 1993).

While air temperature affects primarily photosynthesis, thus indirectly influences N_2 fixation by changing the carbon supply, soil temperature directly affects nodulation and symbiotic activity. Temperatures below 10 °C will generally delay nodulation. As in most biological systems, the bulk of the literature regarding this aspect suggests that each symbiotic system is subject to a specific temperature optimum (e.g., Schomberg and Weaver 1992; Shuler and Hannaway 1993; Zang et al. 1995). While beyond that point water deficits often cause complications, low temperatures usually reduce symbiotic activity due to a reduction in dissimilation of sugars in the nodule. Keeping the obligate exceptions in mind, one can generally say that N_2 fixation is more temperature sensitive than the uptake of combined N.

11.5.2 Soil pH

Under acidic conditions N_2 fixation is in general detrimentally affected, limiting *Rhizobium* survival and persistence in soils, thus reducing nodulation. Failure to nodulate under acid-soil conditions is common. Acid soils may also be low in levels of various plant nutrients, e.g., available calcium, magnesium, phosphorus, and molybdenum. They frequently also contain levels of aluminum and magnesium, which are toxic to the host (Zahran 1999). However, acid-tolerant rhizobial strains and legumes (e.g., Cunningham and Munns 1984; Graham et al. 1994; Hansen 1994; Mpepereki et al. 1997) as well as rhizobial strains with tolerance to high aluminum and magnesium levels have been reported in the literature (Graham 1992). Lime applications to ameliorate soil conditions have been demonstrated to be effective, while calcium fulvate was shown to be a feasible alternative to increase soil pH (Van Der Watt et al. 1991). Even a small increase of soil pH—from 4.5 to 4.9—can have a marked effect on growth and nitrogen fixation (Peoples et al. 1995).

11.5.3 Salinity

Salinity affects plant growth in general and legumes are no exception (Manchanda and Garg 2008). Salinity reduces nodulation due to distortion of root hairs as well as epidermal and hypodermal cells (Sprent and Sprent 1990). However, also mature nodules are affected, and degradation of bacteroids in the host cell can be observed if salinity and reduced photosynthetic activity occur together (James et al. 1993). Also nitrogenase activity is affected, and lower amounts of leghemoglobin, soluble protein, and carbohydrate contents of the cytosol and bacteriods are found under specific conditions (Abdalla 1992). High salt concentrations ultimately lead to reductions in N_2 fixation and eventually its termination, probably due to osmotic withdrawal of water from nodules. For the cultivation of legumes under saline conditions, the selection of resistant rhizobia in combination with tolerant legumes appears to be most promising.

11.5.4 Soil Moisture

Water stress has a profound impact on N_2 fixation. A retardation of nodule development is often observed and attributed to a lower rhizobial motility in the soil, reduced reproduction of bacteria in the rhizosphere, and a reduction of infection thread formation. Established nodules experience premature senescence and may loose their functionality completely, if they experience a loss of 20 % of their fresh weight. Effects can be reduced if drought-tolerant strains are selected

(Athar and Johnson 1996; Esfahani and Mosterjeran 2011; Gehlot et al. 2012; Karmakar et al. 2014). The process of N_2 fixation may decline by the reduction of photosynthate supply by the host plant to the nodules, leading to lower respiration. Due to their persistent meristem, usually indeterminate nodules tend to recover easier after alleviation of drought conditions. In Mediterranean climate of Western Australia, perennial legumes loose some of their nodules during the dry summer period (Hansen and Pate 1987a). Winter rains induce subsequent nodule development and N_2 fixation after some time required for the establishment of a new generation of nodules and the reactivation of some old nodules. At least in the *Acacia* spp. studied in the above investigation, it was conclusively proven that the plant had a higher tolerance to desiccation than the symbiotic system in the nodules.

At the other end of the spectrum, under conditions of water logging, the largest complication legumes have to overcome is the reduction in oxygen supply to the nodules for respiration. Enhanced phellogen activity of nodules and production of particularly large lenticels often occur under such conditions. The development of an aerenchyma tissue is an alternative adaptation, allowing a continuous supply of oxygen for nodule respiration (e.g., *Viminaria juncea*). Stem nodules as found in *Sesbania rostrata* are yet another kind of adaptation to conditions of frequent water logging (see Fig. 11.3).

11.5.5 Presence of Rhizobia for Nodulation

The presence of compatible rhizobia is of crucial significance for the establishment of a functional symbiotic system and N_2 fixation. It is obvious that inoculations are essential in the absence of rhizobia. However, they are equally relevant in the presence of native, inefficient rhizobia. In an agricultural setting, results can be improved by sowing leguminous crops jointly with the provision of a rhizobial inoculum. These need to be optimized in terms of their nodulation and fixation performance under the specific edaphic conditions, taking moisture, texture, pH, salinity, etc. into account. In the USA, Canada, and a number of other countries, such practice is long established. The application of rhizobia relies mostly on two alternative means, i.e., direct soil application or seed-applied inoculation. The former generally relies on granules or liquid culture, which is diluted to a slurry to be distributed by spray application. The latter variant usually requires mixing of seeds with a peat culture—or other carriers—containing the rhizobia (for review see Deaker et al. 2004).

However, it is often a laborious task to optimize the compatibility of the macro- and microsymbiont, since strain characteristics, environmental conditions, resistance to disease, and numerous other factors play an important role. Introduced rhizobia need to survive in the local edaphic conditions, withstand the complete assortment of chemicals in the soils, as well as natural or artificial bactericides. Bacteriophages and competition with other bacteria and diazotrophs, trying to fill the same ecological niche, pose further challenges for the survival of inoculates.

Consequently, inoculation with superior strains does not per se lead to improved N_2 fixation. Therefore, field tests are required to ensure that the inoculation has been successful. Some reports in the literature have also demonstrated a beneficial effect of co-inoculation with rhizobia and mycorrhizae (Wang et al. 2011). Such dual inoculations usually increase the availability of phosphorus to the plant, enhancing plant growth and photosynthate supply to the nodules, in turn leading to an improved N_2-fixation performance.

The improvement of nodulation via the genetic modification of the host plant has been another attempt to improve nodulation and N_2 fixation. Supernodulating mutants lack the internal regulation to control the number of root nodules that harbor the symbiotic diazotrophs. However, increased nodulation is often associated with reductions in plant growth resulting in a disproportion between photosynthetic capacity of the host and the respiratory demands of the nodules (Hansen et al. 1992a, b, 1993a, b; Novak 2010). Despite such potential restrictions, it was recently shown that under optimal growth conditions for supernodulated plants, symbiotic activity of nodules may not be hampered (Cabeza et al. 2014).

11.6 Sustainable Food Security: Are Legume-*Rhizobium* Symbioses a Solution?

In an environmental context, sustainability is viewed as the ability to continue a defined behavior indefinitely. Morelli (2011) defines environmental sustainability more precisely: "as meeting the resource and services needs of current and future generations without compromising the health of the ecosystems that provide them—and more specifically—as a condition of balance, resilience, and interconnectedness that allows human society to satisfy its need while neither exceeding the capacity of its supporting ecosystems to continue to regenerate the services necessary to meet those needs nor by our actions diminishing biological diversity." In view of the developments outlined in the introduction, it becomes clear that the unquestionable benefits of the "Green Revolution" led primarily to a vast increase in provision of agricultural products, i.e. food to alleviate famines and help to reduce the suffering of the needy. With less than half of the global human population in the 1940s than today, the question of sustainability was less on the mind of people. Now—about 70 years later—Earth supports a lot more inhabitants due to the availability of food in larger quantities. Progress with the reduction of infectious diseases helped further to support the increase in human population to the extent that even the vastly improved food availability is not sufficient to feed everyone. Thus, famines still occur. Agriculture cannot keep up with the growing demand. Logistics of food distribution and storage remain a challenge. So, did the "Green Revolution" solve the problem? One might argue that it appeared so for some time, but essentially it brought the system to a different—higher—level, and with it, the problems grew as well. The intensive application of nitrogenous fertilizer causes

not only environmental problems with the water table through leaching an nitrate contamination, soil degradation, and air pollution but also generates a large climate gas footprint, contributing to global warming. Therefore, modern agriculture—in its intensive form—is not fulfilling the above criteria of sustainability (Bohlool et al. 1992; Morelli 2011).

Biological nitrogen fixation is certainly a better option, if emphasis is prioritized toward sustainability rather than maximum agricultural output (Peoples and Craswell 1992). Due to the high costs of nitrogenous fertilizer, lower-income economies are disadvantaged. Biological nitrogen fixation relies on renewable solar energy, avoids or at least reduces the risks of groundwater pollution, and largely prevents erosion *via* the provision of a vegetative cover. It utilizes a virtually unlimited source of atmospheric N_2 and is a preferred choice in low-income countries, if less intensive agriculture is practiced. The full potential of less known legumes is by far not utilized yet (Sprent et al. 2010). Hence, despite numerous challenges under different conditions, the rhizobial symbioses allow in general a sustainable agricultural production system (e.g., Peoples et al. 2009; Sprent et al. 2010). Further improvements of the symbiotic system—*via* breeding, molecular biological techniques, genetic engineering, or optimizing inoculants and host plants—are likely to further improve the fixation performance of legumes in the field. However, it is unlikely that improvements can match the annual increase of nitrogenous fertilizer of 2–8 % (FAO 2012). Consequently, sustainable food security is possible to achieve only in a situation of a more or less stable demand situation rather than in situations of unchecked dynamic growth. In a stable setting, rhizobial symbioses may well be a solution to achieve food security.

11.7 Conclusion

At the turn of the century, Paul Crutzen and Eugene Stoermer minted the term "Anthropocene" (from the Greek word *ánthrōpos* human being and *cene* meaning new), signifying the dominant role of human beings on altering the environmental conditions on the Earth while rivaling global geophysical processes. Many stratigraphers criticize the term from a geological perspective saying that there is no exact boundary where it appears in the rock strata to differentiate it from the geological term Holocene (entirely recent), which began after the last ice age, 11,700 years ago. However, the term has been well understood, if one looks at it from a wider—not purely geological—perspective. What Crutzen and Stoermer meant to underline is that human societies have for about 200 years fundamentally altered the living conditions on the planet. Climate change, rising sea level, and CO_2 concentrations are just some indicators for these changes. The human alteration of the nitrogen cycle is another important and significant global change of conditions (Vitousek et al. 1997). Heavy application of industrially produced nitrogen fertilizer is one of the factors leading to the departure from sustainable plant production. It has increased agricultural production dramatically during the

last 70 years or so, but it comes with a cost. Input efficiency of N fertilizer is very low, leading to numerous environmental challenges (Vitousek et al. 1997). Consequently, alternatives to intensive N fertilizer use need to be considered. Biological N_2 fixation offers under certain conditions an economically attractive and environmentally sustainable option to reduce external input and the establishment of a balanced production system. The legume-*Rhizobium* system is—apart from the *Azolla*/*Anabaena* symbiotic system in paddy rice cultivation—a major source of combined N in many terrestrial cropping systems. However, modern agriculture is based on maximum output, thereby not taking into account the eventual depletion of agricultural soil, water pollution, fossil fuel consumption, environmental quality, and the effect on global warming. Hence, the real expenses—the environmental costs—are ignored in most intensive agricultural systems. Consequently, irrespective of intensive research, resulting in substantial improvements of host plants and bacterial inoculum, the overall proportion of global contributions of nitrogen fixation relative to industrially fixed N has decreased over the last 40 years. Although exact data are lacking, the majority of publications indicate that the proportion of biologically fixed N_2 has dropped from ⅔ of the global contribution to ½ in the last four decades, which coincides with a continuous increase in global N fertilizer demand of between 2 and 8 % per year (FAO 2012). The improvements of the legume-*Rhizobium* symbiotic system can most likely provide food security in line with the carrying capacity for our species on the globe, but cannot keep up with accelerating expansion of the global population and the *per capita* demand. If we progress further toward a system aiming for the maximization of global human population, i.e., entering deeper into the "Anthropocene" (Crutzen and Stoermer 2000), we may not be able to prevent changing the Earth's environmental system to a state, which cannot support the living conditions existing species are adapted to.

References

Abdalla MH (1992) Nodulation and nitrogen fixation in faba bean (*Vicia faba* L) plants under salt stress. Symbiosis 12:311–319

Athar M, Johnson DA (1996) Influence of drought on competition between selected *Rhizobium meliloti* strains and naturalized soil rhizobia in alfalfa. Plant Soil 184:231–241

Bergman B, Rai AN, Johansson C, Soderback E (1992) Cyanobacterial-plant symbioses. Symbiosis 14:61–81

Boddey RM, Peoples MB, Palmer B, Dart PJ (2000) Use of the ^{15}N natural abundance technique to quantify biological nitrogen fixation by woody perennials. Nutr Cycl Agroecosyst 57:235–270

Bohlool BB, Ladha JK, Garrity DP, George T (1992) Biological nitrogen fixation for sustainable agriculture: a perspective. Plant Soil 141:1–11

Boussingault J (1838) Recherches chimiques sur la vegetation enterprises dans le but d'examinersi les plantes prennent de l'azote de l'atmosphere. Ann Chim Phys 67:1–54

Bowen HJM (1960) Biological fractionation of isotopes. Int J Appl Radiat Isot 7:261–272

Burris RH (1980) The global nitrogen budget—science or séance? In: Newton WE, Orme-Johnson WH (eds) Nitrogen fixation. University Park Press, Baltimore, pp 7–16

Burris RH, Roberts GP (1993) Biological nitrogen fixation. Annu Rev Nutr 13:317–335

Cabeza RA, Lingner A, Liese R, Sulieman S, Senbayram M, Tränkner M, Dittert K, Schulze J (2014) The activity of nodules of the Supernodulating Mutant Mt_{sunn} is not limited by photosynthesis under optimal growth conditions. Int J Mol Sci 15:6031–6045

Calvert HE, Pence MK, Peters GA (1985) Ultrastructural ontogeny of leaf cavity trichomes in *Azolla* implies a functional role in metabolic exchange. Protoplasma 129:10–27

Crutzen PJ (1970) The influence of nitrogen oxides on the atmospheric ozone content. Q J R Meteorol Soc 96:320–325

Crutzen PJ (2008) N_2O release from agro-biofuel production negates global warming reduction by replacing fossil fuels. Atmos Chem Phys 8:389–395

Crutzen PJ, Stoermer EF (2000) The "anthropcene". IGBP Newsl R Swed Acad Sci 41:17–18

Cunningham SD, Munns DN (1984) The correlation between extracellular polysaccharide production and acid tolerance in *Rhizobium*. Soil Sci Soc Am J 48:1213–1226

Deaker R, Roughley RJ, Kennedy IR (2004) Legume seed inoculation technology—a review. Soil Biol Biochem 36:1275–1288

Delwiche CC (1970) The nitrogen cycle. Sci Am 223:136–146

Epping B, Hansen AP, Djalali B, Martin P (1994) Symbiotic efficiency of four *Phaseolus vulgaris* genotypes after inoculation with different strains of *Rhizobium* under controlled conditions. Z Naturforsch 49c:343–351

Esfahani MN, Mosterjeran A (2011) Rhizobial strain involvement in symbiosis efficiency of chickpea–rhizobia under drought stress: plant growth, nitrogen fixation and antioxidant enzyme activities. Acta Physiol Plant 33:1075–1083

Falkowski PG (1997) Evolution of the nitrogen cycle and its influence on the biological sequestration of CO_2 in the ocean. Nature 387:272–275

FAO (2012) Current world fertilizer trends and outlook to 2016. p 43

Galloway JN, Schlesinger WH, Levy H, Michaels A, Schnoor JL (1995) Nitrogen fixation: atmospheric enhancement—environmental response. Glob Biogeochem Cycles 9:235–252

Gehlot HS, Panwar D, Tak N, Tak A, Sankhla IS, Poonar N, Parihar R, Shekhawat NS, Kumar M, Tiwari R, Ardley J, James EK, Sprent JI (2012) Nodulation of legumes from the Thar desert of India and molecular characterization of their rhizobia. Plant Soil 357:227–243

Gehring C, Vlek PLG (2004) Limitations of the ^{15}N natural abundance method for estimating biological nitrogen fixation in Amazonian forest legumes. Basic Appl Ecol l5:567–580

Giller KE, Merckx R (2003) Exploring the boundaries of N_2-fixation in cereals and grasses: a hypothetical and experimental framework. Symbiosis 35:3–17

Graham PH (1992) Stress tolerance in *Rhizobium* and *Bradyrhizobium*, and nodulation under adverse soil conditions. Can J Microbiol 38:475–484

Graham PH, Draeger K, Ferrey ML, Conroy MJ, Hammer BE, Martinez E, Naarons SR, Quinto C (1994) Acid pH tolerance in strains of *Rhizobium* and *Bradyrhizobium*, and initial studies on the basis for acid tolerance of *Rhizobium tropici* UMR1899. Can J Microbiol 40:198–207

Hansen AP (1994) Symbiotic N_2 fixation of crop legumes. Margraf Verlag, Weikersheim, p. 248

Hansen AP, Pate JS (1987a) Comparative growth and symbiotic performance of seedlings of *Acacia* spp. in defined pot culture or as natural understorey components of a eucalypt forest ecosystem in SW Australia. J Exp Bot 38:13–25

Hansen AP, Pate JS (1987b) Evaluation of the ^{15}N natural abundance method and xylem sap analysis for assessing N_2 fixation in understorey legumes in jarrah (*Eucalyptus marginata* Donn ex Sm.) forest in S.W. Australia. J Exp Bot 38:1446–1458

Hansen AP, Pate JS, Atkins CA (1987) Relationship between acetylene reduction, hydrogen evolution and nitrogen fixation in nodules of *Acacia* spp: experimental background to assaying fixation by acetylene reduction under field conditions. J Exp Bot 38:1–12

Hansen AP, Yoneyama T, Kouchi H (1992a) Short term nitrate effects on hydroponically grown soybean cv. Bragg and its supernodulating mutant. I. Carbon, nitrogen and mineral element distribution, respiration and effect of nitrate on nitrogenase activity. J Exp Bot 43:1–7

Hansen AP, Yoneyama T, Kouchi H (1992b) Short term nitrate effects on hydroponically grown soybean cv. Bragg and its supernodulating mutant. II. Distribution and respiration of recently fixed ^{13}C-labelled photosynthate. J Exp Bot 43:9–14

Hansen AP, Yoneyama T, Kouchi H, Martin P (1993a) Respiration and nitrogen fixation of hydroponically cultured *Phaseolus vulgaris* L cv OAC Rico and a supernodulating mutant. Growth, mineral composition and effect of sink removal. Planta 189:538–545

Hansen AP, Yoneyama T, Kouchi H, Hiraoka K (1993b) Respiration and nitrogen fixation of hydroponically cultured *Phaseolus vulgaris* L cv OAC Rico and a supernodulating mutant. 2. Distribution and respiration of recently fixed ^{13}C labeled photosynthate and the effect of sink removal on carbon partitioning. Planta 189:546–556

Hansen AP, Rerkasem B, Lordkaew S (1995) Does ammonium uptake influence xylem sap composition in *Phaseolus vulgaris* L. and *Glycine max* (L) Merril? Experientia 51:1085–1089

Hardarson G, Danso SKA (1993) Methods for measuring biological nitrogen fixation in grain legumes. Plant Soil 152:19–23

Hellriegel H, Wilfarth H (1888) Untersuchungen über die Stickstoff-Nahrung der Gramineen und Leguminosen. Zeitschrift des Vereins für die Rübenzucker-Industrie des Deutschen Reiches. pp 1–234

Herridge DF, Peoples MB, Boddy RM (2008) Global inputs of biological nitrogen fixation in agricultural systems. Plant Soil 311:1–18

Hinnemann B, Nørskov JK (2006) Catalysis by enyzmes: the biological ammonia synthesis. Top Catal 37:55–70

Hirsch AM (1992) Transley Review No 40. Developmental biology of legume nodulation. New Phytol 122:211–237

Hungria M, Franco AA, Sprent JI (1993) New sources of high-temperature tolerant rhizobia for *Phaseolus vulgaris* L. Plant Soil 149:95–102

Iqbal R, Mahmood A (1992) Structural studies on root nodules of *Leucaena leucocephala* with particular reference to the infection process. Pak J Bot 24:142–152

James EK, Sprent JI, Hey GT, Minchin FR (1993) The effect of irradiance on the recovery of soybean nodules from sodium chloride-induced senescence. J Exp Bot 44:997–1005

Joshua DC, Ramani S, Suarez E, Shaikh MS (1992) Growth, stem nodulation, and nitrogen content of *Sesbaniarostrata* plants treated with different rates of urea. J Plant Nutr 15:1353–1358

Karmakar K, Rana A, Rajwar A, Sahgal M, Johri BN (2014) Legume-Rhizobia symbiosis under stress. In: Arora NK (ed) Plant microbes symbiosis: applied facets. Springer, New Delhi, pp 241–258

Kijne JW (1992) The *Rhizobium* infection process. In: Stacey G, Burris RH, Evans HJ (eds) Biological nitrogen fixation. Chapman & Hall, New York, pp 349–398

Kim JS, Rees DC (1992a) Crystallographic structure and functional implications of the nitrogenase molybdenum iron protein from *Azotobacter vinelandii*. Nature 360:553–560

Kim JS, Rees DC (1992b) Structural models for the metal centers in the nitrogenase molybdenum-iron protein. Science 257:1677–1682

Krahn E, Weiss BJR, Kröckel M, Groppe J, Henkel G, Cramer SP, Trautwein AX, Schneider K, Müller A (2002) The Fe-only nitrogenase form *Rhodobacter capsulatus*: identification of the cofactor, an unusual, high-nuclearity iron-sulfur cluster, by Fe *K*-edge EXAFS and ^{57}Fe Mössbauer spectroscopy. J Biol Inorg Chem 7:37–45

Ladha JK, Reddy PM eds (2000) The quest for nitrogen fixation in rice. Proceedings of the third working group meeting on assessing opportunities for nitrogen fixation in rice, 9–12 Aug 1999, Los Baños, Laguna, Philippines. International Rice Research Institute, Makati City, 354pp

Lance C, Rustin P (1984) The central role of malate in plant metabolism. Physiol Veg 22:625–641

Lee CC, Hu Y, Ribbe MW (2010) Vanadium nitrogenase reduces CO. Science 329(5992):642

Long SR (1992) Genetic analysis of *Rhizobium* nodulation. In: Stacey G, Burris RH, Evans HJ (eds) Biological nitrogen fixation. Chapman & Hall, New York, pp 560–597

Manchanda G, Garg N (2008) Salinity and its effects in the functional biology of legumes. Acta Physiol Plant 30:595–618

Matson PA, McDowell WH, Townsend AR, Vitousek PM (1999) The globalization of N deposition: ecosystem consequences in tropical environments. Biogeochemistry 46:67–83
Minchin FR, Witty JF, Sheehy JE, Muller M (1983) A major error in the acetylene reduction assay: decreases in nodular nitrogenase activity under assay conditions. J Exp Bot 34:641–649
Minchin FR, Sheehy JE, Witty JF (1986) Further errors in the acetylene reduction assay: Effects of plant disturbance. J Exp Bot 37:1581–1591
Morelli J (2011) Environmental sustainability: a definition for environmental professionals. J Environ Sustain 1:19–27
Mosier AR, Kroeze C, Nevison C, Oenema O, Seitzinger S, van Cleemput O (1998) Closing the global N_2O budget: nitrous oxide emissions through the agricultural nitrogen cycle. OECD/IPCC/IEA phase II development of IPCC guidelines for national greenhouse gas inventory methodology. Nutr Cycl Agroecosyst 52:225–248
Mpepereki S, Makonese F, Wollum AG (1997) Physiological characterization of indigenous rhizobia nodulating *Vigna unguiculata* in Zimbabwean soils. Symbiosis 22:275–292
Nandi AS, Sen SP (1981) Utility of some nitrogen-fixing microorganisms in the phylosphere of crop plants. Plant Soil 63:465–476
Newton WE (1992) Isolated iron-molybdenum cofactor of nitrogenase. In: Stacey G, Burris RH, Evans HJ (eds) Biological nitrogen fixation. Chapman & Hill, New York, pp 877–929
Novak K (2010) On the efficiency of legume supernodulating mutants. Ann Appl Biol 157:321–342
Orme-Johnson WH (1992) Nitrogenase structure—where to now. Science 257:1639–1640
Ozawa T, Tsuji T (1993) A possible role of the polyamines in the repression of growth of *Bradyrhizobium japonicum* bacteroids in soybean nodules. Plant Cell Physiol 34:899–904
Peoples MB, Craswell ET (1992) Biological nitrogen fixation: investments, expectations and actual contribution to agriculture. Plant Soil 114:13–39
Peoples MB, Herridge DF (1990) Nitrogen fixation by legumes intropical and subtropical agriculture. Adv Agron 44:155–221
Peoples MB, Boddey RM, Herridge DF (2002) Quantification of nitrogen fixation. In: Leigh GJ (ed) Nitrogen fixation at the millennium. Elsevier, Amsterdam, pp 357–389
Peoples MB, Brockwell J, Herridge DF, Rochester IJ, Alves BJR, Urquiaga S, Boddy RM, Dakora FD, Bhattarai S, Maskey SL, Sampet C, Rerkasem B, Khan DF, Hauggaard-Nielsen H, Jensen ES (2009) The contributions of nitrogen-fixing crop legumes to the productivity of agricultural systems. Symbiosis 48:1–17
Peoples MB, Ladha JK, Herridge DF (1995) Enhancing legume N_2 fixation through plant and soil management. Plant Soil 174:83–101
Prather M, Ehhalt D (2001) Atmospheric chemistry and greenhouse gases. In: Houghton JT, Ding Y, Griggs DJ, Noguer M, van der Linden PJ, Dai X, Maskell K, Johnson CA (eds) Climate change. The scientific basis. Cambridge University Press, Cambridge, pp 239–287
Rehder D (2000) Vanadium nitrogenase. J Inorg Biochem 80:133–136
Schöllhorn R, Burris RH (1967) Acetylene as a competitive inhibitor of N_2 fixation. Proc Natl Acad Sci USA 58:213–216
Schomberg HH, Weaver RW (1992) Nodulation, nitrogen fixation, and early growth of arrowleaf clover in response to root temperature and starter nitrogen. Agric J 84:1046–1050
Sebastian J, Chandra AK, Kolattukudy PE (1987) Discovery of a cutinase-producing *Pseudonomas* sp. cohabiting with an apparently nitrogen-fixing *Corynebacterium* sp. in the phyllosphere. J Bacteriol 169:131–136
Shrivastava S, Prasad R, Varma A (2014) Anatomy of root from eyes of a microbiologist. In: Morte A, Varma A (eds) Root engineering. Springer, Berlin, pp 3–22
Shuler PE, Hannaway DB (1993) The effect of preplant nitrogen and soil temperature on yield and nitrogen accumulation of alfalfa. J Plant Nutr 16:373–392
Simpson FB, Burris RH (1984) A nitrogen pressure of 50 atmospheres does not prevent evolution of hydrogen by nitrogenase. Science 224:1095–1097
Singh A, Agarwal M (2008) Acid rain and its ecological consequences. J Environ Biol 29:15–24

Smil V (2011) Nitrogen cycle and world food production (PDF). World Agric 2:9–13
Somasegaran P, Hoben HJ (1994) Handbook of Rhizobia: methods in legume-*Rhizobium* technology. Spinger, Berlin, p. 450
Sprent JI, Sprent P (1990) Nitrogen fixation organisms. Chapman & Hall, London, p. 256
Sprent JI, Odee DW, Dakora FD (2010) African legumes: a vital but under-utilized resource. J Exp Bot 61:1257–1265
Tjepkema JD, Schwintzer CR, Benson DR (1986) Physiology of actinorhizal nodules. In: Briggs WR, Jones RL, Walbot V (eds) Annual review of plant physiology and plant molecular biology. Annual Reviews, Palo Alto, pp 209–232
Towe KM (2002) Evolution of nitrogen fixation. Science 295:798–799
Unkovich MJ, Pate JS (2000) An appraisal of recent field measurements of symbiotic N_2 fixation by annual legumes. Field Crops Res 65:211–228
Van Der Watt HVH, Barnard RO, Cronie IJ, Dekker J, Croft GJB, Van Der Watt MM (1991) Amelioration of subsoil acidity by application of a coal-derived calcium fulvate to the soil surface. Nature 350:146–148
Van Dommelen A, Vanderleyden J (2007) Associative nitrogen fixation. In: Bothe H, Ferguson S, Newton WE (eds) Biology of the nitrogen cycle. Elsevier, Amsterdam, pp 179–192
Vance CP, Heichel GH (1991) Carbon in N_2 fixation: limitations or exquisite adaptation. In: Briggs WR, Jones RL, Walbot V (eds) Annual review of plant physiology and molecular biology. Annual Reviews, Palo Alto, pp 372–392
Vitousek PM, Aber J, Howarth RW, Likens GE, Matson PA, Schindler DW, Schlesinger WH, Tilman GD (1997) Human alterations of the global nitrogen cycle: causes and consequences. Issues Ecol 1:1–15
Wang X, Pan Q, Chen F, Yan X, Liao H (2011) Effects of co-inoculation with arbuscular mycorrhizal fungi and rhizobia on soybean growth as related to root architecture and availability of N and P. Mycorrhiza 21:173–181
Werner D (1992) Physiology of nitrogen-fixing legume nodules. In: Stacey G, Burris RH, Evans HJ (eds) Biological nitrogen fixation. Chapman & Hill, New York, pp 399–431
Wood M, Cooper JE (1988a) Acidity, aluminium and multiplication of *Rhizobium trifolii*: Effects of the initial inoculum density and growth phase. Soil Biol Biochem 20:83–87
Wood M, Cooper JE (1988b) Acidity, aluminium and multiplication of *Rhizobium trifolii*: Effects of temperature and carbon source. Soil Biol Biochem 20:89–93
Zahran HH (1999) *Rhizobium*-Legume symbiosis and nitrogen fixation under severe conditions and in an arid climate. Microbiol Mol Biol Rev 63:968–989
Zang F, Lynch DH, Smith DL (1995) Impact of low root temperature in soybean [*Glycine max* (L.) Merr.] on nodulation and nitrogen fixation. Environ Exp Bot 35:279–285

Chapter 12
Tools and Techniques to Study Multidrug Transporters of Yeasts

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Abstract Considering the increase in incidence of multidrug resistance (MDR) in yeast, there is, in parallel, spurt in research in the field. Expectedly there are several good articles which highlight the MDR research, but there is very limited information available which deals with the methods used to study MDR in yeast. In this contribution, an attempt is made to describe in detail routine protocols used to study different aspects of MDR in yeast, particularly dealing with multidrug efflux pump protein-related functions. Different drug susceptibility assays can be performed on solid or on liquid media using clinically approved protocols. Moreover, properties and activity scales of drug efflux pumps can be measured by performing their substrate efflux activity using suitable substrate depending on choice of protocol or transporter under study. These drug transporters can be overexpressed in different systems or can be expressed in drug-susceptible strains to clearly follow their contribution in development of drug resistance. It is hoped that this chapter will be useful in studying the role of drug transporters in the phenomenona of MDR in yeast.

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12.1 Introduction

Multidrug resistance (MDR) is a phenomenon wherein a resistant organism possesses many different mechanisms, which contribute to enhance collateral drug resistance to variety of xenobiotics. Hence, MDR leads to higher drug tolerance, which is manifested by multiple mechanisms. Many opportunistic fungal pathogens also develop tolerance to different antifungal treatment regimes leading to the development of MDR (White et al. 1998; Shapiro et al. 2011; Prasad et al. 2014).

Among several mechanisms of MDR which have been characterized in yeast includes the development of yeast cell's inability to accumulate drugs intracellularly to toxic levels. This generally happens due to an overexpression of membrane-embedded transporters acting as multidrug efflux pumps. The rapid efflux in resistant strains ensures that the drug is not accumulated to lethal levels (Cannon et al. 2009). Several azole-resistant clinical isolates of pathogenic yeast *Candida albicans* as well as of other fungal pathogens like *Aspergillus fumigates* and *Cryptococcus neoformans* display transcriptional activation of efflux pump-encoding genes and often show reduced intracellular accumulation of drugs, thus confirming the role of efflux proteins in drug extrusion and tolerance (Nascimento et al. 2003; Prasad and Kapoor 2005; Cannon et al. 2009). Additionally, permeability changes leading to decreased drug import also contribute toward to the development of drug resistance (Mansfield et al. 2010).

This chapter focuses on the methods to study the efflux transporters involved in the development of MDR in yeasts, taking example of a pathogenic yeast *C. albicans*. A brief description of efflux pump proteins is preceding methods.

12.1.1 MDR Transporters

An overexpression of efflux pump proteins, particularly ATP-binding cassette (ABC) multidrug transporter proteins Cdr1 and Cdr2 or major facilitator superfamily (MFS) protein Mdr1, is observed in azole-resistant clinical isolates of *C. albicans* (White et al. 2002; Karababa et al. 2004; Kusch et al. 2004; Prasad and Kapoor 2005). Drug-resistant *Candida* cells, which show enhanced expression of efflux pump-encoding genes, also show simultaneous increase in the efflux of drugs (Cannon et al. 2009). Notably, *Candida* genome possesses several members of ABC and MFS family of proteins; however, only a few members of these families function as multidrug transporter with their defined roles in clinical drug resistance (Prasad and Goffeau 2012).

Topologically, both ABC and MFS drug transporters differ from each other and that also reflect different mechanisms of drug transport employed by the members of these two superfamilies of proteins. For instance, full-length ABC transporters generally consist of two similar halves each in turn consisting of one transmembrane domain (TMD) and one cytoplasmic nucleotide-binding domain (NBD).

NBDs that bind and hydrolyze ATP power the substrate efflux, bound within TMDs where drug binding sites exist. Each TMD consists of six continuous alpha-helical transmembrane segments (TMS), arranged to form drug binding sites (Prasad and Goffeau 2012). MFS transporters on the other hand also consist of two similar TMDs each consisting of six TMSs within a single polypeptide chain; however, they are devoid of NBDs. The MFS proteins with role in clinical drug resistance function as drug/H^+ antiporters (Stephanie et al. 1998). The MFS transporter in *Candida*, Mdr1p, is an antiporter and possesses a conserved antiporter motif, critical for its functionality (Pasrija et al. 2007).

Transport of drugs mediated by multidrug transporters belonging to either ABC or MFS superfamilies represents one of the major mechanisms of MDR in *Candida*. The discussion below details methods employed to evaluate drug susceptibility, drug transport, and approaches to study the structure and function of transporter proteins.

12.2 Drug Susceptibility Assay

Antifungal susceptibility testing (AST) has become important criteria to evaluate the increasing incidence of fungal infections particularly due to *C. albicans* and non-albicans species in immune-compromised patients. Susceptibility testing needs to be performed for infectious organisms for which susceptibility to antifungal drugs is not known or for the organisms which are believed to have developed drug resistance through the involvement of one or more resistance mechanisms (CLSI 2008a). It is also imperative to perform susceptibility testing for novel antifungal agents for which earlier reports are not available to get an idea about the range of drug concentrations that can be effective against particular fungal species. Susceptibility test needs to be performed individually for different species, rather than using a source of infection containing multiple fungal species which thus cannot guarantee the results about a particular fungal species (CLSI 2008a).

There are many susceptibility tests developed over the years to monitor development of tolerance to several antifungal agents. It is therefore reasonable to look for reproducible and reliable testing methods to analyze the susceptibility pattern of various fungal species and efficacy of different classes of antifungal drugs. AST is continuously under development since the 1980s. Clinical Laboratory Standards Institute (CLSI) is holding important role in the development and uniformity of the susceptibility testing protocols worldwide. In addition to CLSI, there are many different laboratory and/or industry-developed manual protocols, which add to the antifungal susceptibility testing regimes available to clinicians or researchers to access the antifungal susceptibility patterns (Fothergill 2012; Amsden 2015). CLSI has now given the standard and reliable protocols that are being used for drug susceptibility testing, and results are considered good enough for clinicians for disease follow-up and treatment (Fothergill 2012). This antifungal susceptibility testing can be performed by different standard methods as discussed below and is an

important criterion in guiding the management of antifungal therapy. These methods being used currently differ either on the basis of type of fungi under study or on the basis of experimental procedure being followed. The methods described below are mostly used for planktonic fungal cells particularly *Candida*, but can be extended to other cells with minor modifications. For other filamentous fungi, the methods described may differ, and those are well described elsewhere (Fothergill 2012).

12.2.1 Broth Microdilution Method

Minimum inhibitory concentration (MIC), which is routinely used in describing drug susceptibility of an organism, implies a minimum drug concentration, which causes the specified reduction in the microbial growth by microdilution assay. MIC determination is routinely employed as an upfront procedure to evaluate drug susceptibility of particular species. For the determinations of MICs, currently two different CLSI protocols are released and being followed which include M27-A3 (CLSI 2008a) for broth dilution yeast antifungal susceptibility testing and M38-A2 (CLSI 2008b) for broth dilution filamentous fungi antifungal susceptibility testing. Current M27-A3 antifungal susceptibility testing of yeasts is specially evaluated for *Candida* and *Cryptococcus* species which generally cause invasive infections but has been also used extensively for other dimorphic fungi (most fungi are dimorphic and can exist either exclusively as yeast or in mycelial forms or as mixed populations) (Fothergill 2012).

Some of the important parameters set for the current protocol for MIC determinations include inoculum size of $0.5–2.5\times10^3$ CFU/ml, RPMI 1640 as testing media, and incubation time of 24, 48, or 72 h depending on species and/or drug in use.

MIC determinations are generally done in 96-well MIC plates (traditionally used for ELISA), which gives the option to test the range of drug concentrations in a single run. Earlier, five-point breakup criteria were used to depict the efficacy of an antifungal drug against fungal isolates. These include susceptible, intermediate, dose-dependent susceptible, resistant, and non-susceptible; however, routinely, only two-point breakup criteria are considered for fungal isolates (Fig. 12.1a). Thus a strain could either be susceptible or non-susceptible against the tested drug. An end point can be evaluated visually (by looking at the growth density) and subsequently can be validated spectrophotometrically. The time to terminate incubation of MIC assay could vary but should be uniformly followed for set of determinations. Normally, the species under consideration used to be major factor in deciding the incubation time. For example, for *Candida* species, it is generally 48 h, and in case of *Cryptococcus* species, 72 h is recommended. In addition to species under consideration, different drugs in use could also be a factor in deciding the incubation period. For example, for amphotericin B, fluconazole, and candins, 24 h incubation period is recommended for end point determination, while it is

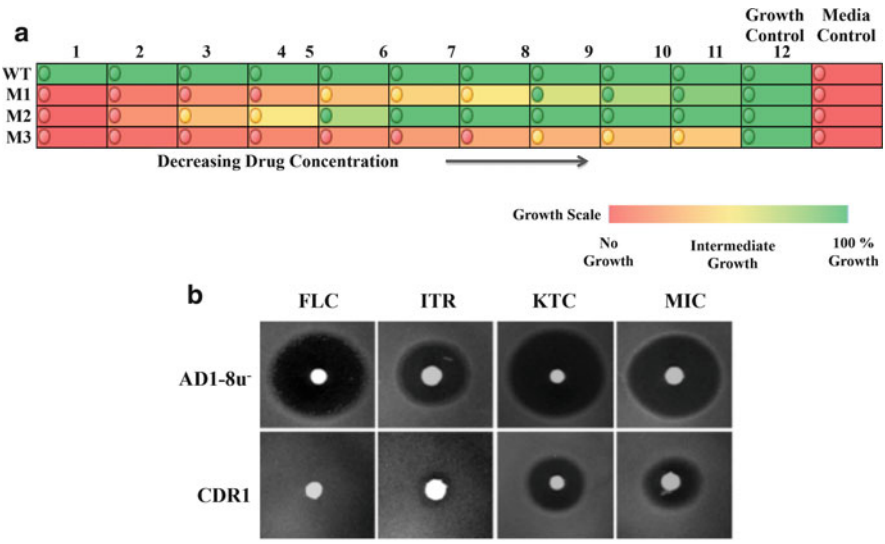

Fig. 12.1 (**a**) Depiction of results of broth microdilution assay of WT yeast strain and compared with three different mutants (M1–M3). Concentration of drug decreases twofold each time from well 1–10. Well 11 represents growth control and does not contain any drug, while well 12 represents media control without drug and cells. Growth increases from *red* to *green color* as shown in growth scale. (**b**) Drug resistance profile of AD1-8u^{-} and CDR1 against particular concentration of antifungal drugs fluconazole (FLC) 64 μg, itraconazole (ITC) 4 μg, ketoconazole (KTC) 2 μg, and miconazole (MIC) 0.25 as seen in disk diffusion assay

48 or 72 h for other drugs in case of *Cryptococcus neoformans*. For *Candida* species, end point for candins is 24 h time point, while for amphotericin B and fluconazole, end point can be read at 24 or 48 h (CLSI 2008a; Fothergill 2012).

12.2.2 Disk Diffusion Method

In addition to broth microdilution assay, an alternative (CLSI M44A2) methodology for drug susceptibility testing is also recommended. This is considered as cost-effective and rapid antifungal susceptibility testing protocol for microbiology laboratories. The disk diffusion protocol was earlier adopted by CLSI for antibacterial testing and has now also been recommended for antifungal susceptibility testing (NCCLS 2004; CLSI 2009). The CLSI document provides approved breakpoints for *Candida* species for antifungal azole drugs like fluconazole and voriconazole and to candins like caspofungin and provides quantitative results after 20–24 h incubation (CLSI 2009). To assist with yeast growth and to enhance visualization of the diameters of zone of inhibition, the addition of methylene blue glucose is encouraged (Fig. 12.1b). This document is further expected to

encourage the development of disk diffusion testing for additional antifungal agents and other fungal pathogens (CLSI 2009).

For non-dermatophyte filamentous fungi, the current CLSI protocol for disk diffusion antifungal susceptibility testing is M51-A (CLSI 2010). By using this protocol, susceptibility to antifungal drugs like itraconazole, posaconazole, voriconazole, amphotericin B, and caspofungin has been evaluated. Epidemiological cutoff values (ECVs) have been developed based on a comparison of zone diameters vs. minimal inhibitory concentrations (MICs) or minimal effective concentrations (MECs). ECVs have been used to detect those isolates that are likely to have acquired enhanced resistance or reduced susceptibility to antifungal agents. In this particular protocol, results can be determined after only 16–48 h incubation as opposed to 24–72 h with CLSI document M38 (CLSI 2010). This document is further expected to encourage the development of disk diffusion testing for other antifungal agents.

12.2.3 Checkerboard or Synergy Assays

Checkerboard assays between two different antifungal compounds are performed to address their interaction and are the basis for calculation of the fractional inhibitory concentration index (FICI) (Odds 2003; Septama and Panichayupakaranant 2016). One of the drugs at a non-inhibitory concentration is diluted by twofold dilutions along the x-axis of the 96-well plates, while another drug at its non-inhibitory concentration is diluted by twofold dilutions along the y-axis of the plate. Notably, individual drug should not be inhibitory to growth at any of the concentrations used to check for synergistic effects with other compound. After inoculation of about 100 µl of 0.1×10^5 cells in each well, the plates are incubated at 30 °C for 48 h in RPMI 1640 media. The MIC value is considered as the lowest concentration of the compounds, alone or in combination, required for inhibition of fungal growth measured at O. D_{500} nm (Maurya et al. 2011; Septama and Panichayupakaranant 2016). The interaction between the two drug compounds is determined by quantifying the FICI using the following equation:

$$\mathrm{FICI} \quad = \mathrm{FIC_A}+\mathrm{FIC_B}= {\mathrm{C_A}}^{\mathrm{comb}}/{\mathrm{MIC_A}}^{\mathrm{alone}}+{\mathrm{C_B}}^{\mathrm{comb}}/{\mathrm{MIC_B}}^{\mathrm{alone}},$$

where ${\mathrm{MIC_A}}^{\mathrm{alone}}$ and ${\mathrm{MIC_B}}^{\mathrm{alone}}$ are the MICs of drug A and B when acting alone and ${\mathrm{C_A}}^{\mathrm{comb}}$ and ${\mathrm{C_B}}^{\mathrm{comb}}$ are concentrations of drugs A and B at the iso-effective combinations, respectively (Maurya et al. 2011). If the condition arises that the MIC or MEC results in an off-scale value, the value is converted to the next higher or lower concentration. Growth and sterility controls need to be included in each individual experiment/plate. If any solvent is used for drug suspension, the equal amount of solvent should also be checked individually for each strain to exclude any solvent-related effects on growth leading to misinterpretation. A particular drug

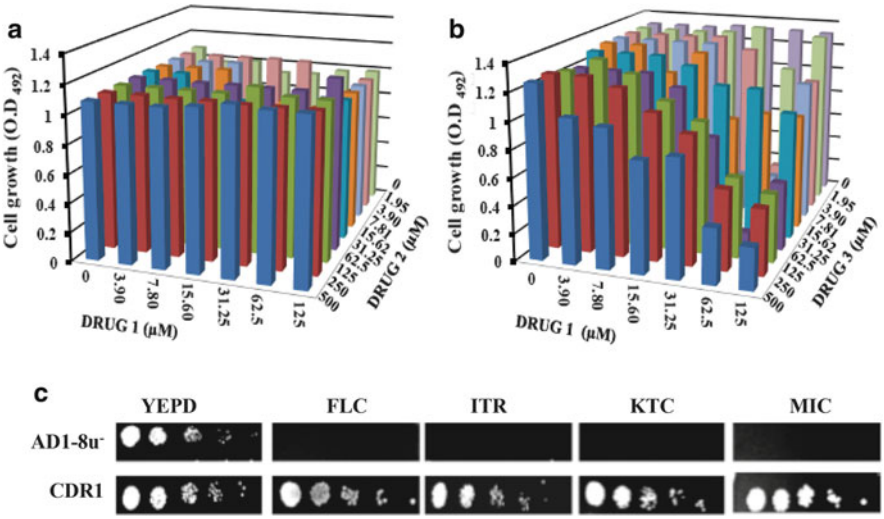

Fig. 12.2 (**a**) Checkerboard assays depicting synergy between drug 1 and drug 3, whereas no synergy is observed between drug 2 and drug 1. The minimum inhibitory concentration of drug 1 which was 250 μM became 125 μM in the presence of drug 3 (62.5 μM), while no change in MIC is observed in drug 1 and 2 combination treatment. (**b**) Drug susceptibility spot assay showing drug resistance profile of AD1-8u$^-$ (control) cells and CDR1 as drug-resistant cells expressing drug transporter Cdr1, against the indicated drugs at following concentrations. Fluconazole (FLC), 5 μg/ml; itraconazole (ITC), 2 μg/ml; ketoconazole (KTC), 0.15 μg/ml; and miconazole (MIC), 0.16 μg/ml. The CDR1 cells are resistant to indicated drugs at given concentration as compared to AD1-8u$^-$ control cells

is considered as synergistic when FICI $\leq$ 0.5, FICI of $>$0.5 and $\leq$4.0 = no interaction, and FICI of $>$4.0 = antagonism (Odds 2003). As an example results of checkerboard assay showing no interaction between drug 1 and drug 2 and synergy between drug 1 and drug 3 are shown in Fig. 12.2a, b.

12.2.4 Serial Dilution Assay

Serial dilution assay or drug susceptibility spot assays are performed routinely in microbiology laboratories to evaluate the antifungal effects of drugs or susceptibility pattern of different yeast strains on solid agar drug plates. Serial dilution or spot assay is generally performed when the MIC of a particular drug against a particular fungal strain is already known. In cases where MIC of a compound is not known, broth microdilution method could be used by trying out range of concentrations. The method is good when one is interested in comparing growth of set of isolates, which may be expected to have altered susceptibility. Susceptibility of test strains is then determined by employing known drug concentrations, and growth is compared with their respective isogenic parental strains by serial dilution assays

(Mukhopadhyay et al. 2002; Vandeputte et al. 2012). Protocols for testing antifungal susceptibility include the following general steps.

- Cultures are grown overnight either in YEPD medium (containing yeast extract, 5 g/l; peptone, 10 g/l; and glucose, 20 g/l) or on YNB media supplemented with 2 % glucose and minimum requisite quantities of auxotrophic supplements (if any).
- The cells are then suspended in sterile phosphate-buffered saline (PBS) or 0.9 % normal saline to an $O.D_{600}$ of 0.1.
- Four microliter drops of fivefold serial dilution of each of the yeast strains is spotted on YNB or YEPD agar plates supplemented with different requisite concentrations of drugs
- Drug-free YNB or YEPD agar plates as a growth control are taken for each strain.
- Growth differences in the presence or absence of drugs are recorded for each strain after incubation of agar plates at 30 °C for 48 h.

Fig. 12.2c depicts the results of serial dilution spot assay against different drugs involving resistant and susceptible yeast strains.

12.3 Drug Transport Assays

One of the important mechanisms of drug resistance includes enhanced drug efflux by MDR strains (Cannon et al. 2009). Hence several methods have been developed to study drug extrusion by yeast cells. For measuring drug transport, both the accumulation of drug and its extrusion can be used. The choice of substrates is the key to success of this method. The promiscuous nature of drug transport proteins of both ABC and MFS superfamilies has provided choice of many substrates that could be used, and their transport can be monitored. There are host of compounds that drug efflux proteins can extrude, but for monitoring the transport, substrate could either be fluorescent or radiolabeled. Table 12.1 gives the list of some of the substrates, which can be successfully used to measure drug transport by yeast cells.

12.3.1 Drug Efflux

One of the commonly used approaches to study drug efflux by membrane transporters is the use of colored fluorescent substrates, which can easily be measured either by monitoring fluorescence and/or absorbance. As an example, a protocol for the measurement of absorbance of red-colored rhodamine 6G (R6G) efflux, a fluorescent substrate of *C. albicans* drug transporter Cdr1p, is described which

Table 12.1 List of compounds which can be used for measurement of drug transport and transporter activity

Type of assay	Compound	Properties	References
Efflux	Rhodamine 6G	Fluorescent	Shukla et al. (2006)
	Rhodamine B	Fluorescent	Decottignies et al. (1998)
Accumulation	Nile Red	Fluorescent	Ivnitski-Steele et al. (2009)
	Rhodamine B	Fluorescent	Decottignies et al. (1998)
	Rhodamine 123	Fluorescent	Krishnamurthy et al. (1998a)
	^{3}H-β-estradiol	Radiolabeled	Krishnamurthy et al. (1998b)
	^{3}H-fluconazole	Radiolabeled	Mukhopadhyay et al. (2002) and Esquivel et al. (2015)
	^{3}H-Methotrexate	Radiolabeled	Pasrija et al. (2007)
	^{3}H-Corticosterone	Radiolabeled	Krishnamurthy et al. (1998b)
	^{3}H-Clotrimazole	Radiolabelled	Esquivel et al. (2015)
	^{3}H-Ketoconazole	Radiolabelled	Esquivel et al. (2015)

can be adopted to any cells or system with suitable modifications (Mukhopadhyay et al. 2002; Shah et al. 2015).

- Approximately 1×10^{6} cells from an overnight primary culture grown in a shaking incubator at 30 °C is inoculated in 250 ml of YPD, and this secondary culture is grown further for 5–6 h at 30 °C.
- These exponential phase cells after centrifugation are washed three times with phosphate-buffered saline buffer (PBS).
- The cells are subsequently resuspended as a 2 % cell suspension in de-energization buffer (5 mM dinitrophenol and 5 mM 2-deoxy-D-glucose) and incubated for 45 min at 30 °C. This step ensures depletion of endogenous energy pool, which could hamper accurate transport measurements.
- The cells are then washed, resuspended in PBS containing 2 % glucose, to which R6G is added to a final concentration of 10 μM and incubated.
- Alternatively, cells can also be de-energized by incubating the cells in the absence of glucose for the time period of about 2 h to deplete the cells of energy. This can be achieved by resuspending cells as a 2 % cell suspension in PBS to which rhodamine 6G is added to a final concentration of 10 μM. The cell suspension is incubated for 2 h at 30 °C. The cells after washing are resuspended in PBS with 2 % glucose.
- An aliquot of fixed volume is taken at various times and centrifuged at 9000 g for 2 min.
- The absorbance of the supernatant is routinely measured spectrophotometrically at 527 nm (which coincides with the absorption maxima of R6G). The gradual increase in absorbance reflects efflux amount of R6G mediated by the transporter protein. A typical example of R6G efflux measurement as a function of time of ABC fungal transporter is shown in Fig. 12.3a.

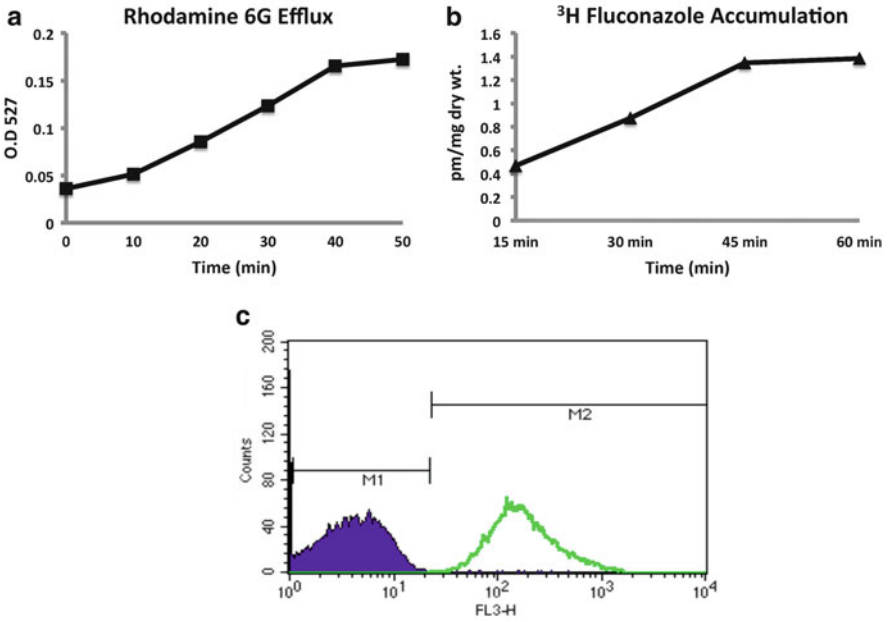

Fig. 12.3 (**a**) Typical representation of a time course of R6G transport, measured as the intensity (absorbance) of the substrate effluxed out in the extracellular media at different intervals of time. (**b**) Time course of radiolabeled fluconazole (^{3}H-FLU) accumulation in a yeast strain. As routine measurements, uptake can be measured at a given time point of incubation showing maximum accumulation. For instance, in the given figure, 45 or 60 min of incubation time can be routinely considered as good end point for the measurement of accumulation of radiolabeled fluconazole. (**c**) Nile red accumulation by test (*purple*, M1 quadrant) and control cells (*green line*, M2 quadrant) as measured and analyzed by flow cytometry. Test sample contains an active transporter leading to efflux of substrate and thus lowers the accumulation of NR, while control sample devoid of transporter accumulates NR and thus shows peak shift toward M2 quadrant due to high fluorescence for same number of cells analyzed as that of test sample

12.3.2 Drug Accumulation

An enhanced drug accumulation in a given population of cells would imply that cells are unable to efflux the drug efficiently leading to increase in intracellular concentration of it. Hence, the measurement of accumulation of drug also provides a good indirect measure of efflux activity. Although both efflux and accumulation monitor the drug transport activity of membrane proteins, sometimes the two cannot be used interchangeably. Since, it may not be always possible to monitor the efflux substrate because they get diluted in extracellular media. This is particularly the case with radiolabeled substrates. In such cases, it is better to measure the accumulation of the substrates, which is a good indirect measure of efflux activity of the cell. Moreover, some of the fluorescent compounds like Nile red (NR) fluoresce better in hydrophobic microenvironments, and thus its accumulation

inside the cells can be easily measured compared to its presence in extracellular media (Ivnitski-Steele et al. 2009; Mandal et al. 2012).

Some of the commonly used radiolabeled molecules which have been used as substrates of well-known drug transporters of *C. albicans* include ^{3}H-β-estradiol, ^{3}H-fluconazole, ^{3}H-methotrexate, etc. (Krishnamurthy et al. 1998b; Mukhopadhyay et al. 2002; Shah et al. 2015). As an example, the protocols for two substrates ^{3}H-fluconazole (radiolabeled substrate) and NR (fluorescent substrate), commonly employed to study intracellular accumulation and activity of membrane transporters, are described:

^{3}H-FLC Accumulation

- The mid-log phase cells (~10^{7} cells) are washed with PBS at 3220 g for 5 min.
- The cells are then de-energized for 45 min in a de-energizing buffer (as described above). The de-energized cells are washed and resuspended as 5 % cell suspension in YNB media.
- An aliquot of the suspension is incubated with 100 nM of ^{3}H-FLC (specific activity 3–5 Ci/mmol) in the presence of 2 % glucose (as an energy source).
- After particular time of incubation, a fixed aliquot is withdrawn and rapidly filtered and washed twice with ice-cold PBS buffer using a 0.45 μm cellulose nitrate filter disks in a manifold filtration assembly (Millipore, USA). The filtration and washing steps should be completed very rapidly for accurate kinetic measurements. It is recommended to prechill the wash solution before the start of the experiment.
- The washed filter disks are air-dried, and the filter disks are immersed in liquid scintillation cocktail (commercially available). The accumulated radioactivity in cells can be measured in any standard liquid scintillation counter.

As an example, a typical representation of accumulation of radiolabeled fluconazole as a function of time is shown in Fig. 12.3b.

NR Accumulation

- An overnight grown culture either in YEPD medium (containing yeast extract, 5 g/l; peptone, 10 g/l; and glucose, 20 g/l) or on YNB media supplemented with 2 % glucose is required as starting material.
- From this overnight culture, cells equivalent to A_{600} of 0.1 O.D are inoculated and grown at 30 °C, in an incubator shaker with shaking at 200 rpm until the A_{600} reaches around 0.25 O.D.
- The exponential phase cells are harvested and diluted as a 5 % cell suspension. The fluorescent NR is added at a final concentration of 7 μM. After rapid mixing, cells are incubated at 30 °C for another 30 min. The point to terminate the reaction may vary with system.
- After incubation of cells at indicated time, the cells are harvested at 3220 g for 4–5 min and resuspended in PBS with 2 % glucose.
- The NR accumulation by different test and control cells is measured and analyzed by flow cytometry employing a good flow cytometer. A typical NR accumulation as seen in flow cytometry is shown in Fig. 12.3c.

12.4 ATPase Assays

Among the multidrug transporters, those belonging to ABC superfamily utilize the energy generated by ATP hydrolysis to efflux out substrates from the cells. Hence, all the ABC proteins known as transport ATPases elicit enzyme activity coupled to drug transport. The measure of ATPase activity thus becomes a good indicator of transport activity of the transporter protein (Higgins 2007).

Many different protocols are being followed for ATPase activity measurement of ABC transporters (Nakamura et al. 2001; Shukla et al. 2006). For yeast cells, which possess cell wall (CW), any method of reliable plasma membrane-ATPase (PM-ATPase) activity measurement should include the removal of CW followed by separation of PM from other cellular components and provide an option to isolate crude membrane (CM) or purified PM preparations.

12.4.1 Preparation of Sample for ATPase Activity

As discussed in the following sections, for both the measure of drug transport and ATPase activities, the transporter protein under investigation should be overexpressed in an established expression system, which would provide enriched cells/membranes for the measurements. A reasonable pure PM preparation is necessary for accurate assessment of ATPase activity of PM-localized transporters. As a routine, however, CM devoid of intercellular organelles can also be used as a starting material for the measurements of activity. Procedures for preparing CM and PM membranes (Monk et al. 1991; Shukla et al. 2003) are briefly discussed below:

Crude Membrane Preparation

- The procedure described here is typically good for CM preparation from 250 ml of overnight culture (starting from 0.3 O.D).
- Harvest the overnight cell cultures (12–14 h, approx. 5 gm wet weight from about 250 ml culture) at 3220 g for 10–15 min.
- Suspend the pellet in 10 ml homogenization buffer (HB), (Table 12.2) after washing 2–3 times in distilled water.

Note: The fraction at this stage can be stored at −80 °C for few days if it cannot be processed immediately.

- Add washed and sterilized glass beads of 3–5 mm size, (typically 3–5 g of glass beads are added to a cell pellet obtained from 250 ml of overnight culture). The cells and glass beads are put through a "cell disruptor" in cold temperatures to disrupt the cells. The cell disruptor can be any mechanical device capable of carrying and breaking cells in controlled cold temperature. Some of the disruptors, which can be employed for disrupting yeast cells generally, include

Table 12.2 Homogenization buffer (HB)

Buffer component	Final concentration
Tris-Cl (pH 7.5)	10 mM
PMSF	100 μg/ml
EDTA	0.5 mM
TPCK	100 μg/ml
TLCK	50 μg/ml
Aprotinin	1–2 μg/ml
Leupeptin	1–2 μg/ml
Pepstatin	1 μg/ml

Braun homogenizer, Fast Prep, vortexer, and pestle/mortal grinding (later two give poor yield because of less sample carrying capacity and often maintenance of cold temperature is compromised).

- Centrifuge the disrupted mixture at 805 g for 10 min in cold (4–6 °C) to pellet down glass beads and unbroken cell debris.
- The supernatant devoid of cell debris and unbroken cells is pelleted by centrifugation at 75000 g.
- The pelleted CM is recovered after carefully decanting off the supernatant, and the pellet is suspended in resuspension buffer (RB) (Table 12.3) containing 10 % glycerol.
- The CM samples at this step can be stored at −80 °C for several weeks before using it for PM preparation and can also be directly used for ATPase assays.

Purified Plasma Membrane Preparation

PM is prepared and recovered by employing density gradient centrifugation. Typically, sucrose gradient (53.5 % and 43.5 %) gives good recovery of PM fraction. The gradient is made by carefully layering sucrose of different density (Fig. 12.4) in prechilled ultracentrifuge tubes. The CM from 250 ml starting culture is carefully overlaid on top of the gradient.

- The centrifuge tube with sucrose gradient and overlaid CM is subjected to ultracentrifugation for about 4–5 h at 100000 g in a swinging bucket rotor at 4 °C.
- The centrifuge tubes are carefully removed from the centrifuge.
- One could clearly see the middle interfacial ring-shaped PM fraction, which can be sucked out carefully and transferred to another tube.
- The withdrawn PM fraction is suspended in RB (5–6 ml) and is solubilized in it by gentle repeated pipetting.
- The solubilized PM fraction is subjected to another round of centrifugation in an ultracentrifuge at 110000 g for 30–50 min at 4 °C.
- The pelleted PM fraction is suspended in appropriate volume (100–500 μl) of RB depending on the required protein concentration for further uses.
- It is recommended to aliquot the PM fraction in small fractions and store at −80 °C for further use. The distribution of PM fraction into smaller portions will minimize repeated cycles of freezing and thawing of PM, which may denature the proteins.

Table 12.3 Resuspension buffer (RB)

Buffer component	Final concentration
Tris-Cl (pH 7.5)	10 mM
Glycerol	10 %
NaCl	150 mM
PMSF	100 µg/ml
TPCK	100 µg/ml
TLCK	50 µg/ml
Aprotinin	1–2 µg/ml
Leupeptin	1–2 µg/ml
Pepstatin	1 µg/ml

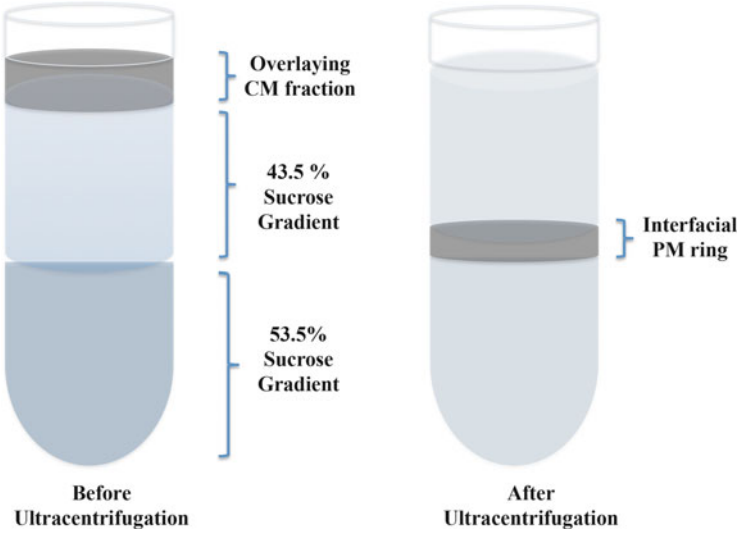

Fig. 12.4 Sucrose density gradient setup to isolate PM fraction. Before ultracentrifugation, discontinuous density gradient is set up by using two sucrose concentrations as shown, 53.5 % and 43.5 % sucrose overlaid with CM fraction. PM fraction, after the centrifugation, can be seen at the interface of the two gradients. The rest of the CM components either settle down as the pellet or scatter across at different density positions

12.4.2 ATPase Activity Measurements

One of the commonly followed colorimetric ATPase assay protocols for membrane transporters of fungal ABC proteins is discussed below (Nakamura et al. 2001; Shukla et al. 2006):

- In a typical 100 µL reaction mixture containing 10 µg PM protein (the protein amount may vary depending upon the transporter protein under study and its enrichment) in an ATPase buffer (60 mM Tris, pH 7.5 and 80 mM $MgCl_2$), 5 mM ATP is added, and mixture is rapidly transferred from ice to a preset 30 °C

water bath. This temperature jump will initiate the enzymatic reaction, which can be followed over a period of time. For initial experiments, a time course of ATP hydrolysis is prepared to obtain a linear relationship between time of incubation and ATP hydrolysis. The protocol described below is terminated at 30 min of incubation.

- The reaction mixture is incubated at 30 °C for 30 min in water bath.
- The reaction is arrested by the addition of 1 ml of stop solution (0.5 % SDS, 2 % H_2SO_4, 0.5 % ammonium molybdate), followed by the addition of 10 μl of freshly prepared 10 % ascorbic acid as coloring reagent.
- The method exploits to measure the release of inorganic phosphate (Pi) as an index of ATPase activity.
- The intensity of blue color indicates the amount of Pi released which could be measured at 750 nm (or a red filter) using a spectrophotometer.

Additional precautions:

1. To eliminate the contribution of nonspecific ATPases like vacuolar and mitochondrial ATPases, the reaction mixture could be supplemented with their specific inhibitors such as 0.2 mM ammonium molybdate, 50 mM KNO_3, and 10 mM NaN_3.
2. ATPase activity of many ABC transporters is specifically sensitive to inhibitors like oligomycin and orthovanadate or to aurovertin B; any of the inhibitors can be added up in the reaction mixture depending on protein under study to get the ATPase activity specific to the protein of interest.

12.5 Overexpression of Transporter Proteins

To study functional aspects of drug transporter proteins that may include assays such as drug susceptibility, drug transport, ATPase and screening of potential inhibitors, etc., it is preferred to have an overexpressed version of protein of interest. Additionally, overexpression systems are also required to obtain purified proteins in biochemical quantities to investigate structure (crystallization) and function of transporters in great details. Given the hydrophobic nature and huge size of majority of transporter proteins, it is often challenging to express and purify it in an active form, which remains a major roadblock. However, there are ample examples of successful methods developed over the years, which can yield purified protein in sufficient quantities for biochemical and structural characterization. Following section describes few overexpression systems, which have been successfully developed and used for the functional studies of multidrug transporter proteins from yeast cells.

12.5.1 Heterologous Overexpression System

Heterologous overexpression of MDR pump proteins of a pathogenic yeast *C. albicans* in *Saccharomyces cerevisiae* has been widely used as a novel tool for the structure-function analyses of these transporters. Such studies unpinned crucial molecular details of drug efflux, protein trafficking, and transport cycle. For instance, one study reported overexpression of ~20 membrane proteins from various prokaryotic and eukaryotic hosts and highlighted advantages and drawbacks of each system (Bernaudat et al. 2011). In another study, 25 human ABC transporters were expressed in yeast *Pichia pastoris* in which 11 transporters demonstrated high expression levels (Chloupkova et al. 2007). For the overexpression of major multidrug transporters of fungal origin, Nakamura et al. (Nakamura et al. 2001) and Decottignies et al. (Decottignies et al. 1998) have developed a heterologous expression system for ABC and MFS transporter proteins (Fig. 12.5). The system uses *S. cerevisiae* AD1-8u$^-$, which is deleted in seven major ABC transporters (Pdr5p, Pdr10p, Pdr11p, Pdr15p, Snq2p, Yor1p, and Ycf1p), as an overexpression host. Owing to the deletion of major drug transporters, AD1-8u$^-$ is highly susceptible to drugs due to their impaired efflux. The AD1-8u$^-$ host also harbors a gain of function mutation *pdr1–3*, which regulates the expression of many ABC transporters like *PDR5, SNQ2, YOR1, PDR10*, *and PDR15* (Decottignies et al. 1998). The gain of function of Pdr1 protein drives high expression of ABC transporters by keeping their promoters in activated form. Nakamura et al. (Nakamura et al. 2001) have constructed a vector pSK-PDR5PPUS where hyperactive PDR5 promoter (because of gain of function mutation of Pdr1) drives high expression of cloned transporter ORF. Recombinant vector having desired transporter is integrated into host AD1-8u$^-$ at *PDR5* locus via homologous recombination leading to single-copy integration with stable expression of the transporter gene of interest. A new derivative vector of pSK-PDR5PPUS named pABC3, which promotes hyperexpression of chosen transporter, is also available [reviewed in Niimi et al. (2005)]. This vector also carries the property of *PDR5* promoter-driven expression of protein and has 8 bp cutting rare restriction enzyme sites for *Pac I and Not I* at multiple cloning sites, which makes it unique to clone any desired gene.

Transformation cassette having *PDR5* promoter, cloned gene of interest, *PGK1* terminator, *URA3* marker, and 3′ end of *PDR5* ORF can be obtained by digestion at *Asc I* enzymatic site. This cassette can be used to integrate gene of interest at genomic DNA of AD1-8u$^-$ at *PDR5* locus for stable hyperexpression. Cannon's group using the same overexpression system characterized three classes of fungal membrane proteins, ABC transporters Pdr5p, CaCdr1p, CaCdr2p, CgCdr1p, CgPdh1p, CkAbc1p, and CneMdr1p, MFS transporter CaMdr1p, and the cytochrome P450 enzyme CaErg11p (Lamping et al. 2007). This heterologous expression system has been successfully used in characterization of various multidrug transporters mainly in the context of substrate specificity, molecular mapping of drug binding sites, NTPase activity, posttranslational modification, screening of pump blockers, and functional vs. structural analysis [reviewed in Niimi et al. (2005) and Rawal et al. (2013)]. In spite of heterologous expression system giving insightful contributions, artifactual

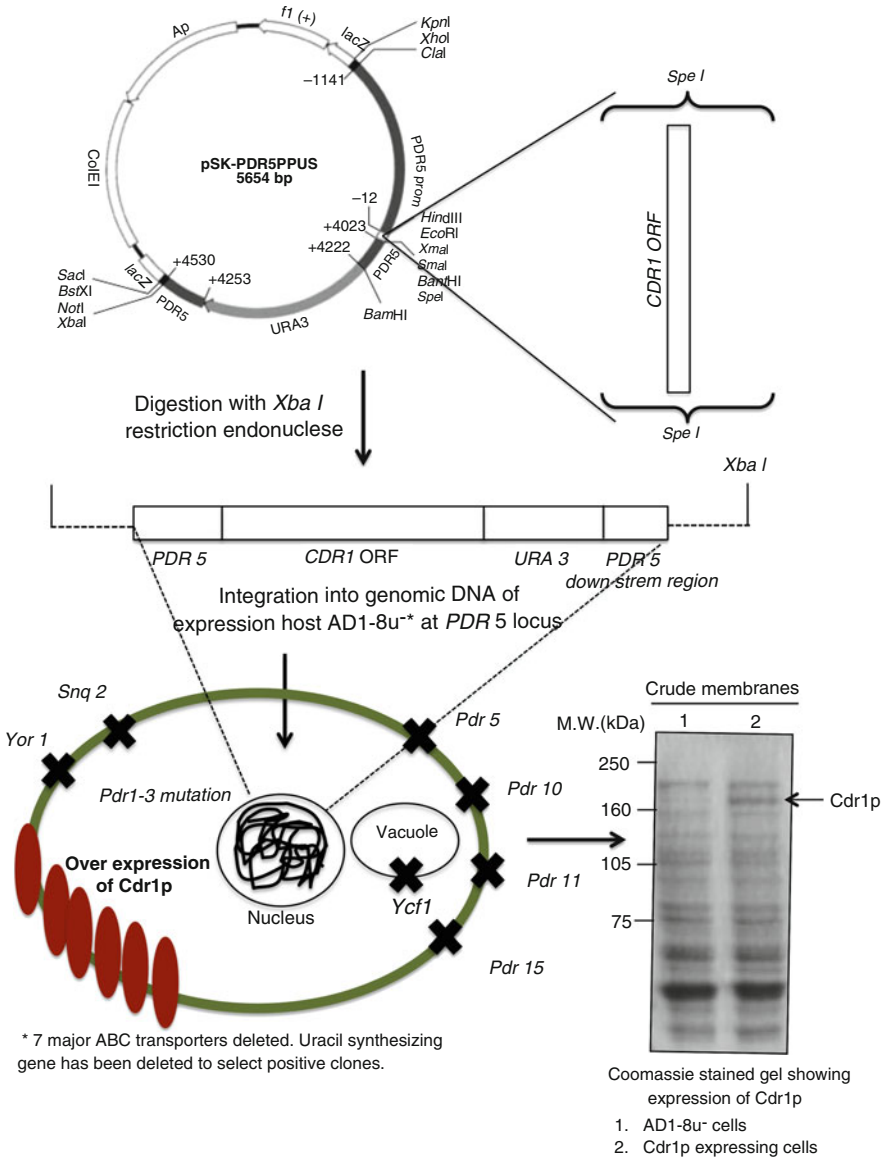

Fig. 12.5 Picture demonstrating strategy for the heterologous expression of multidrug ABC transporter Cdr1p of *C. albicans*. Amplification of *CDR1* is done using forward and reverse primers containing *Spe I* site from the genomic DNA of *C. albicans*. Amplicon is ligated at *Spe I* site of the vector pSK-PDR5PPUS

concerns associated with a heterologous background could not be ignored and should be kept in consideration. Therefore, to circumvent artifactual effects of a heterologous system, there is always need for the development of an in vivo expression system for the overexpression of multidrug transporter proteins.

Protocol for Integration of Cdr1 in AD1-8u$^-$ Expression Host

The following solutions are required: All the solutions should be filter sterilized.

Lithium acetate mix (LAM): 10 ml of 10× TE + 10 ml of 1M lithium acetate + 80 ml water

PEG mix: 8 ml of 50 % PEG-3350 + 1 ml of 10× TE+ 1 ml of 1M lithium acetate

10× TE: 10 ml of 1M Tris (pH: 7.5) + 2 ml of 0.5 M EDTA + 88 ml of water

1. Inoculate 5 ml YPD media with fresh colony of AD1-8u$^-$ cells, and allow it to grow at 30 °C overnight under rotation.
2. Inoculate 50 ml of YPD media with 500 µl of overnight grown culture, and let the OD_{600} reach to ~0.6–0.8.
3. Harvest the growing cells and wash with 10 ml of filter-sterilized LAM.
4. Suspend cells in 0.5 ml of LAM and leave it for 1 h at room temperature on rotation.
5. Take 200 µl of cells, and add ~5 µg (in 10 µl) of linearized pSK-PDR5PPUS-CDR1 (tagged /untagged) plasmid with *XbaI* (gel purified) and 10 µl (10 mg/ml) of sheared salmon sperm DNA (heated at 90 °C for 10 min followed by vortexing and then plunge into ice).
6. Incubate at room temperature for 30 min under rotation.
7. Add 1 ml of PEG mix and then leave it at room temperature for about 45 min followed by 10 min at 42 °C.
8. Spin down the cells, and resuspend in 200 µl of TE buffer and plate 100 µl on uracil-negative plate. Keep the plate in incubator at 30 °C. White colonies should appear after 2–3 days of incubation.
9. Pick few well-grown colonies, and check the expression of Cdr1p using crude (CM) or plasma membrane (PM). Single-copy integration should be confirmed by Southern blotting/PCR.

12.6 Tagging of MDR Proteins

12.6.1 Fluorescent Tagging

Expression of tagged protein has been very useful tool to address various important questions about general understanding of the functioning of drug transporters. Fluorescent tags like green fluorescent protein (GFP), red fluorescent protein (RFP), cyan fluorescent protein (CFP), and yellow fluorescent protein (YFP) have been successfully exploited to understand the dynamics, trafficking, folding, and functioning of various ABC transporters. However, since these tags may interfere with proper folding, trafficking, and localization and may impact its function, negative effects of tagging should always be kept in mind. Prasad and his group

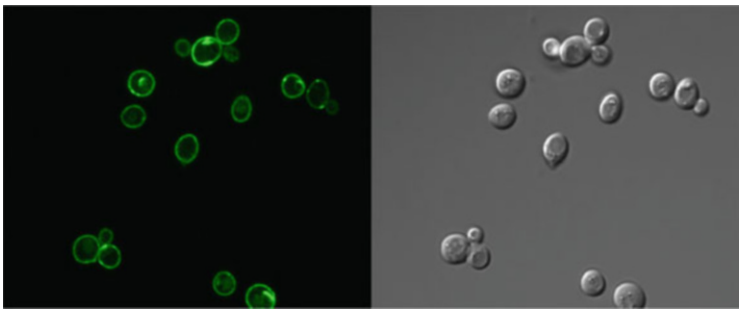

Fig. 12.6 Confocal image (*left panel*) showing expression and localization of GFP-tagged Cdr1p onto the plasma membrane. Expression of GFP-tagged Cdr1p was achieved using expression system developed by Nakamura et al. (2001). The *right panel* depicts phase contrast image of the *left panel*

successfully expressed functionally active, C terminally tagged GFP-fused version of Cdr1p (Cdr1p-GFP) (Fig. 12.6) and Mdr1p (Mdr1p-GFP) (Shukla et al. 2003; Pasrija et al. 2007) using pSK-PDR5PPUS vector or GFP-fused version of *Candida* QDR transporters in pABC3-GFP vector (Shah et al. 2014) and integrated them in *S. cerevisiae* AD1-8u^{-} for detailed characterization. Taken together, fluorescent tagging serves a powerful tool to capture key events inside live cells in real time deciphering mechanistic secrecies of functioning and dynamics of MDR proteins.

12.6.2 FLAG/His Tagging

Many instances, smaller non-fluorescent tags such as FLAG (DYKDDDDK) and poly-histidines are preferred due to possible obstruction by bigger fluorescent tags in enzyme kinetics and assays with purified recombinant protein. Among fungal ABC transporters, Pormorski and group (Marek et al. 2011) successfully purified yeast ABC transporter Aus1, which is required for sterol uptake using FLAG tag and assessed the effect of various lipids on its activity with purified reconstituted protein. Shukla et al. have purified recombinant Cdr1p as His-Cdr1p by employing heterologous overexpression system developed by Nakamura et al. (Shukla et al. 2006). Purified protein could be successfully used for the structural and functional analysis by exploiting intrinsic fluorescence of the protein. Cannon's group successfully overexpressed ABC transporters Pdr5p, CaCdr1p, CaCdr2p, CgCdr1p, CgPdh1p, CkAbc1p, and CneMdr1p, MFS transporter CaMdr1p, and the cytochrome P450 enzyme CaErg11p as EGFP, RFP, FLAG, hexahistidine, and tetracystein-tagged proteins in heterologous system, developed by his group (Lamping et al. 2007) for functional characterization and screening of compounds with potential antifungal activities.

12.6.3 Functional Reconstitution of Purified Cdr1p

There are very limited studies depicting purification of fungal drug transporters with functional reconstitution. Yeast ABC mitochondrial transporter Atm1p has been characterized using proteoliposomes (Kuhnke et al. 2006). Another yeast ABC transporter Aus1 has also been reconstituted into liposomes for functional characterization (Marek et al. 2011). The following is a general protocol for reconstitution of purified Cdr1p. This protocol will serve as a template for the reconstitution of other fungal drug transporters.

Strategy to Purify and Reconstitute ABC Transporter Cdr1p

1. Grow *S. cerevisiae* AD1-8u$^-$ cells expressing Cdr1p-$(His)_6$ fusion protein till late exponential phase (~5 × 10^7 cells/ml). Disrupt the cells in 50 ml falcon tubes and suspend in homogenization buffer (50 mM Tris pH 7.5, 2 mM $MgCl_2$) and protease inhibitors (add PMSF, TPCK, TLCK, aprotinin, leupeptin, and pepstatin at concentrations mentioned in Section 12.4.1) at 4 °C using glass beads with intermittent vigorous vortexing. Remove unbroken cells and debris by centrifugation at 1000 g at 4 °C for 10 min. Prepare CM by ultracentrifugation at 1,00,000 g for 1 h at 4 °C.
2. Suspend pelleted CM in resuspension buffer (20 mM Tris pH 7.5, 2 mM $MgCl_2$, 150 mM NaCl, 20 % glycerol, 20 mM imidazole) followed by solubilization with 1 % (w/v) Triton X-100 for about 30 min at 4 °C.
3. Spin sample at 18,000 g at 4 °C for about 45 min. Collect supernatant avoiding surface lipid layer. Allow supernatant to bind with Ni-NTA agarose resin (Qiagen) (pre-equilibrated with 20 mM Tris and 0.05 % Triton X-100) for 2 h in rotation at 4 °C.
4. Wash resin with buffer containing 20 mM Tris (pH 7.5), 150 mM NaCl, 2 mM $MgCl_2$, 0.1% (w/v) Triton X-100, 30 mM imidazole, and protease inhibitors (as described above) followed by elution with buffer containing 50 mM Tris (pH 7.5), 150 mM NaCl, 2 mM $MgCl_2$, 0.05 % (w/v) Triton X-100, 500 mM imidazole, and 10 % glycerol.
5. Dialyze the sample for 12 h at 4 °C against the buffer having 50 mM Tris (pH 7.5), 30 mM NaCl, 2 mM $MgCl_2$, and 0.05 % (w/v) Triton X-100.
6. To achieve higher purity, eluted sample can be subjected to one round of anion-exchange chromatography using Q sepharose resin pre-equilibrated with 50 mM Tris (pH 7.5) and 0.01 % (w/v) Triton X-100, and allow it to bind for 1 h at 4 °C. Wash resin with buffer having 50 mM Tris (pH 7.5), 2 mM $MgCl_2$, 50 mM NaCl, and 0.1 % (w/v) Triton X-100, and elute protein with buffer containing Tris 20 mM (pH 7.5), 2 mM $MgCl_2$, 200 mM NaCl, and 0.1 % (w/v) Triton X-100.
7. Purity of the protein can be assessed by densitometric analysis using Image J software of silver-stained sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) run gel. Activity of purified Cdr1p can be estimated using same protocol as mentioned in Section 12.4.2. Cdr1p-specific activity in purified sample should be assessed in the presence of 50 μM oligomycin, which generally shows 80–90 % decrease in the ATPase activity.

Functional Reconstitution of Purified Cdr1p

Cdr1p is able to translocate phosphoglycerides from inner monolayer to outer monolayer of membrane lipid bilayer (termed as floppase activity) (Shukla et al. 2007). The floppase activity of Cdr1p can be measured experimentally in functionally reconstituted system (Fig. 12.7). Below is a typical protocol to measure floppase activity of Cdr1p.

1. Take ~4 μM of egg phosphatidylcholine solution (in chloroform 100 mg/ml) in glass tube, and dry it under stream of N_2. Dissolve dried sample in 500 μl of reconstitution buffer containing 20 mM Tris (pH 7.5), 2 mM $MgCl_2$, and 2 % (w/v) Triton X-100 till solution becomes clear.
2. Add 500 μl of purified protein (20–50 μg) followed by the addition of 100 mg of freshly prepared SM2 Bio-Beads (washed with methanol then by 20 mM Tris pH 7.5, 2 mM $MgCl_2$ and 100 mM NaCl), and keep under rotation on Roto-Torque (Cole-Parmer Instrument Company, LLC.) for 3 h at room temperature. Leave it in rotation for ~20 h at 4 °C. Increasing turbidity of the solution represents formation of proteoliposomes.
3. This method of reconstitution generally gives Cdr1p to incorporate in inside-out (IO) orientation (ATP-binding site facing out). As depicted in Fig. 12.7a, in IO orientation of Cdr1 reconstitution, one would measure reverse phosphoglyceride translocase activity (outer monolayer to inner monolayer of lipid bilayer).
4. Dissolve fluorescently labeled lipids NBD-PC (1-acyl-2-[1-[(7-nitro-2,1,3-benzoxadiazol-4-yl)-amino]dodecanoyl]-*sn*-glycero-3-phosphocholine) (Fig. 12.7a) or NBD-PS (1-palmitoyl-2-[6-[(7-nitro-2,1,3-benzoxadiazol-4-yl) amino]-caproyl]-*sn*-glycero-3-phosphoserine) in transport buffer [10 mM Tris-Cl, 200 mM sucrose and 5 mM MgCl2 (pH 7.5)] at 1 mg/ml concentration.
5. Take 100 μl of proteoliposomes and make up to 2 ml with transport buffer. Add labeled lipid to final concentration of 1.5 μM.
6. Incubate sample for 10 min at room temperature to allow labeled lipid to incorporate onto outer leaflet (Fig. 12.7b).
7. To initiate lipid transport, add 50 μl of ATP and ATP regenerating system. Allow the reaction to happen at 30 °C in dark for about 30–90 min. Upon completion of reaction, add 5 mM dithionite to quench fluorescence of NBD lipids on outer leaflets.
8. Monitor total fluorescence (excitation 470 nm and emission 540 nm) of the sample, which would reflect sum of movement of labeled lipids from outer membrane to inner membrane mediated by reconstituted protein. Figure 12.7c depicts a typical reconstitution experiment and its readout.

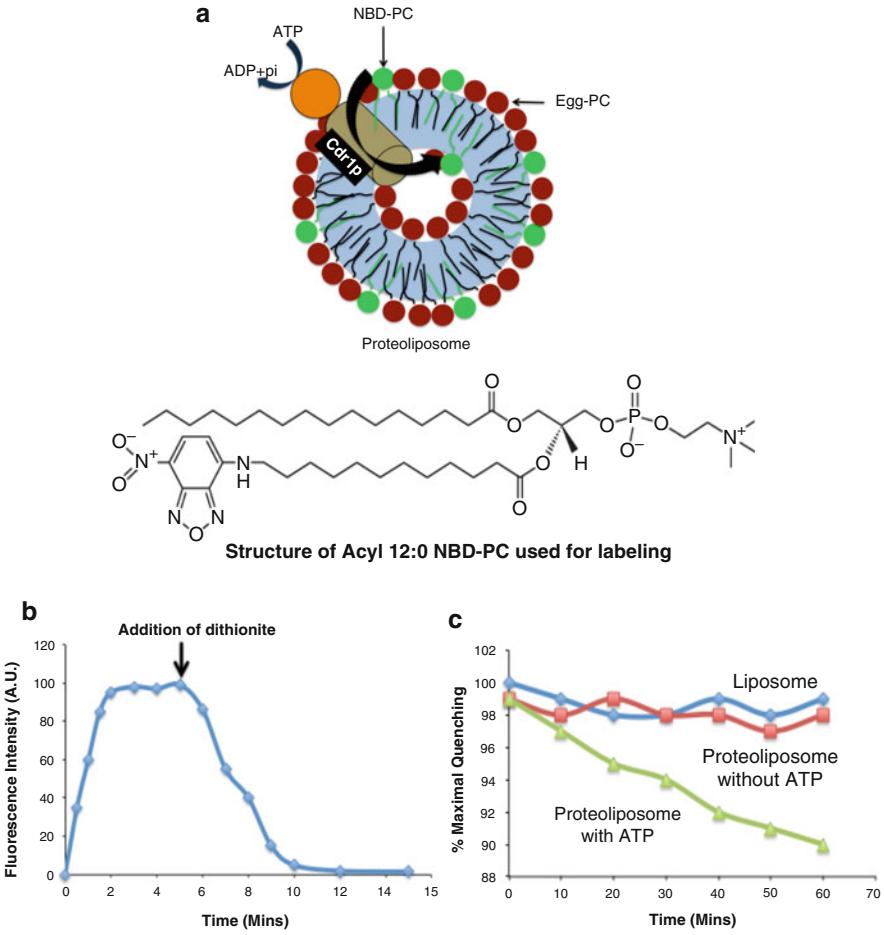

Fig. 12.7 (**a**) Diagram showing Cdr1p incorporated into liposomes in inside-out orientation and mediating translocation of labeled lipid NBD-PC (*green color*) from outer leaflet to inner leaflet upon hydrolysis of ATP. Lower panel shows structure of NBD-PC used for labeling and analyzing Cdr1p-mediated translocase activity. (**b**) Labeling of proteoliposomes: Proteoliposomes are incubated with labeled lipid NBD-PC in transport buffer for indicated time durations to allow its incorporation on to outer leaflet of lipid bilayer. Incorporation of NBD-PC on to outer leaflet is assessed by significant increase in fluorescence intensity (excitation 470 nm and emission 540 nm) within 3–5 min. Addition of 5 mM dithionite results in >98 % drop in the fluorescence within 5 min, which demonstrates proper labeling of liposomes with NBD-PC. (**c**) Proteoliposomes reconstituted with 20 μg of purified Cdr1p demonstrate ~10 % (*green*) protection at 60 min after the initiation of translocase reaction, from dithionite-mediated quenching of fluorescence of NBD-PC due to movement from outer layer to inner layer. Only liposomes (*blue*) and proteoliposomes without source of energy (−ATP) (*red*) are considered as negative controls

12.7 Concluding Remarks

The contribution, which is not exhaustive, has summarized most common methods used to study drug susceptibility in yeast cells. Many instances, a specific example of a protocol specific to cell type protein is discussed to highlight detailed steps involved therein. The information provided is expected to help researchers dealing with drug resistance and drug transporters in yeast cells, but these can be adopted to other microbial system particularly belonging to fungi as well with suitable modifications.

References

Amsden JR (2015) Fungal biomarkers, antifungal susceptibility testing, and therapeutic drug monitoring—practical applications for the clinician in a tertiary care center. Curr Fungal Infect Rep 9:111–121

Bernaudat F, Frelet-Barrand A, Pochon N, Dementin S, Hivin P et al (2011) Heterologous expression of membrane proteins: choosing the appropriate host. PLoS One 6:e29191

Cannon RD, Lamping E, Holmes AR, Niimi K, Baret PV et al (2009) Efflux-mediated antifungal drug resistance. Clin Microbiol Rev 22:291–321

Chloupkova M, Pickert A, Lee JY, Souza S, Trinh YT et al (2007) Expression of 25 human ABC transporters in the yeast *Pichia pastoris* and characterization of the purified ABCC3 ATPase activity. Biochemistry 46:7992–8003

CLSI (2010) Method for antifungal disk diffusion susceptibility testing of non dermatophyte filamentous fungi; approved guideline. CLSI document M51-A. Clinical and Laboratory Standards Institute, Wayne

CLSI (2008a) Reference method for broth dilution antifungal susceptibility testing of yeasts; approved standard-third edition. CLSI document M27-A3. Clinical and Laboratory Standards Institute, Wayne

CLSI (2008b) Reference method for broth dilution antifungal susceptibility testing of filamentous fungi; approved standard CLSI document M38-A2. Clinical and Laboratory Standards Institute, Wayne

CLSI (2009) Method for antifungal disk diffusion susceptibility testing of yeasts; approved guideline. 2nd edn. CLSI document M44-A2. Clinical and Laboratory Standards Institute, Wayne

Decottignies A, Grant AM, Nicholas JW, de Wet H, McIntosh DB et al (1998) ATPase and multidrug transporter activities of the overexpressed yeast ABC protein Yor1p. J Biol Chem 273:12612–12622

Esquivel BD, Smith AR, Zavrel M, White TC (2015) Azole drug import into the pathogenic fungus *Aspergillus fumigatus*. Antimicrob Agents Chemother 59:3390–3398

Fothergill A (2012) Antifungal susceptibility testing: Clinical Laboratory and Standards Institute (CLSI) methods. In: Hall GS (ed) Interactions of yeasts, moulds, and antifungal agents. Humana Press, Totowa, NJ, pp 65–74

Higgins CF (2007) Multiple molecular mechanisms for multidrug resistance transporters. Nature 446:749–757

Ivnitski-Steele I, Holmes AR, Lamping E, Monk BC, Cannon RD et al (2009) Identification of Nile red as a fluorescent substrate of the *Candida albicans* ATP-binding cassette transporters Cdr1p and Cdr2p and the major facilitator superfamily transporter Mdr1p. Anal Biochem 394:87–91

Karababa M, Coste AT, Rognon B, Bille J, Sanglard D (2004) Comparison of gene expression profiles of *Candida albicans* azole-resistant clinical isolates and laboratory strains exposed to drugs inducing multidrug transporters. Antimicrob Agents Chemother 48:3064–3079

Krishnamurthy S, Chatterjee U, Gupta V, Prasad R, Das P et al (1998a) Deletion of transmembrane domain 12 of CDR1, a multidrug transporter from *Candida albicans*, leads to altered drug specificity: expression of a yeast multidrug transporter in baculovirus expression system. Yeast 14:535–550

Krishnamurthy S, Gupta V, Snehlata P, Prasad R (1998b) Characterisation of human steroid hormone transport mediated by Cdr1p, a multidrug transporter of *Candida albicans*, belonging to the ATP binding cassette super family. FEMS Microbiol Lett 158:69–74

Kuhnke G, Neumann K, Muhlenhoff U, Lill R (2006) Stimulation of the ATPase activity of the yeast mitochondrial ABC transporter Atm1p by thiol compounds. Mol Memb Biol 23:173–184

Kusch H, Biswas K, Schwanfelder S, Engelmann S, Rogers PD, Hecker M et al (2004) A proteomic approach to understanding the development of multidrug-resistant *Candida albicans* strains. Mol Genet Genomics 271:554–565

Lamping E, Monk BC, Nimmi K, Holmes AR, Tsao S et al (2007) Characterization of three classes of membrane proteins involved in fungal azole resistance by functional hyperexpression in *Saccharomyces cerevisiae*. Eukaryot Cell 6:1150–1165

Mandal A, Kumar A, Singh A, Lynn AM, Kapoor K et al (2012) A key structural domain of the *Candida albicans* Mdr1 protein. Biochem J 445:313–322

Mansfield BE, Oltean HN, Oliver BG, Hoot SJ, Leyde SE, Hedstrom L et al (2010) Azole drugs are imported by facilitated diffusion in *Candida albicans* and other pathogenic fungi. PLoS Pathog 6:e1001126

Marek M, Milles S, Schreiber G, Daleke DL, Dittmar G et al (2011) The yeast plasma membrane ATP binding cassette (ABC) transporter Aus1 purification, characterization and the effects of lipids on its activity. J Biol Chem 286:21835–21843

Maurya IK, Pathak S, Sharma M, Sanwal H, Chaudhary P et al (2011) Antifungal activity of novel synthetic peptides by accumulation of reactive oxygen species (ROS) and disruption of cell wall against *Candida albicans*. Peptides 32:1732–1740

Monk BC, Kurtz MB, Marrinan JA, Perlin DS (1991) Cloning and characterization of the plasma membrane H-ATPase from *Candida albicans*. J Bacteriol 173:6826–6836

Mukhopadhyay K, Kohli A, Prasad R (2002) Drug susceptibilities of yeast cells are affected by membrane lipid composition. Antimicrob Agents Chemother 46:3695–3705

Nakamura K, Nimmi M, Holmes AR, Yates JE, Decottignies A et al (2001) Functional expression of *Candida albicans* drug efflux pump Cdr1p in a *Saccharomyces cerevisiae* strain deficient in membrane transporters. Antimicrob Agents Chemother 45:3366–3374

Nascimento AM, Goldman GH, Park S, Marras SA, Delmas G et al (2003) Multiple resistance mechanisms among *Aspergillus fumigatus* mutants with high-level resistance to itraconazole. Antimicrob Agents Chemother 47:1719–1726

NCCLS (2004) Reference method for antifungal disk diffusion susceptibility testing of yeasts; approved guideline. NCCLS document M44-A. National Committee for Clinical Laboratory Standards, Wayne

Niimi M, Wada S, Tanbe K, Kaneko A, Takano Y et al (2005) Functional analysis of fungal drug efflux transporters by heterologous expression in *Saccharomyces cerevisiae*. Jpn J Dis 58:1–7

Odds FC (2003) Synergy, antagonism, and what the chequerboard puts between them. J Antimicrob Chemother 52:1. doi:10.1093/jac/dkg301

Pasrija R, Banerjee D, Prasad R (2007) Structure and function analysis of CaMdr1p, a major facilitator superfamily antifungal efflux transporter protein of *Candida albicans*: identification of amino acid residues critical for drug/H+ transport. Eukaryot Cell 6:443–453

Prasad R, Goffeau A (2012) Yeast ATP-binding cassette transporters conferring multidrug resistance. Annu Rev Microbiol 66:39–63

Prasad R, Kapoor K (2005) Multidrug resistance in yeast *Candida*. Int Rev Cytol 242:215–248

Prasad R, Shanh AH, Dhamgaye S (2014) Mechanisms of drug resistance in fungi and their significance in biofilms. In Antibiofilm agents. Springer series on biofilms, vol 8, pp 45–

Rawal MK, Khan MF, Kapoor K, Goyal N, Sen S et al (2013) Insight into pleiotropic drug resistance ATP-binding cassette pump drug transport through mutagenesis of Cdr1p transmembrane domains. J Biol Chem 288:24480–24493

Septama AW, Panichayupakaranant P (2016) Synergistic effect of artocarpin on antibacterial activity of some antibiotics against methicillin-resistant *Staphylococcus aureus, Pseudomonas aeruginosa,* and *Escherichia coli*. Pharm Biol 54:686–691

Shah AH, Rawal MK, Dhamgaye S, Komath SS, Saxena AK et al (2015) Mutational analysis of intracellular loops identify cross talk with nucleotide binding domains of the yeast ABC transporter Cdr1p. Sci Rep 5:11211. doi:10.1038/serp11211

Shah AH, Singh A, Dhamgaye S, Chauhan N, Suneetha KJ et al (2014) novel role of a family of major facilitator transporters in biofilm development and virulence of *Candida albicans*. Biochem J 460:223–235

Shapiro RS, Robbins N, Cowen LE (2011) Regulatory circuitry governing fungal development, drug resistance, and disease. Microbiol Mol Biol Rev 75:213–267

Shukla S, Rai V, Banerjee D, Prasad R (2006) Characterization of Cdr1p, A major multidrug efflux protein of *Candida albicans*: purified protein is amenable to intrinsic fluorescence analysis. Biochemistry 45:2425–2435

Shukla S, Rai V, Saini P, Banerjee D, Menon AK et al (2007) Cdr1p, a major multidrug ABC transporter of *Candida albicans*, translocates fluorescent phospholipids in a reconstituted system. Biochemistry 46:12081–12090

Shukla S, Saini P, Smriti JS, Ambudkar SV et al (2003) Functional characterization of Candida albicans ABC transporter Cdr1p. Eukaryot Cell 2:1361–1375

Stephanie SP, Paulsen IT, Saier MH Jr (1998) Major facilitator superfamily. Microbiol Mol Biol Rev 62:1–34

Vandeputte P, Pradervand S, Ischer F, Coste AT, Ferrari S et al (2012) Identification and functional characterization of Rca1, a transcription factor involved in both antifungal susceptibility and host response in *Candida albicans*. Eukaryot Cell 11:916–931

White TC, Holleman S, Dy F, Mirels LF, Stevens DA (2002) Resistance mechanisms in clinical isolates of *Candida albicans*. Antimicrob Agents Chemother 46:1704–1713

White TC, Marr KA, Bowden RA (1998) Clinical, cellular, and molecular factors that contribute to antifungal drug resistance. Clin Microbiol Rev 11:382–402

Chapter 13
Approaches for Determining Antimicrobial Drug-Resistant Bacteria: The Way Ahead

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Abstract Since the beginning, medical practitioners and veterinarians have employed a vast variety of antimicrobials to treat microbial infectious diseases that were based primarily on past clinical experiences. However, the emergence of resistance among microbial species against traditionally used antimicrobials has made it more difficult for clinicians to empirically select an appropriate antimicrobial agent. As a result, global concern has been deviated toward finding the efficacy of available antimicrobials. Therefore, it is recommended to validate already existing in vitro antimicrobial susceptibility testing (AST) methods. Although a variety of methods exist, the goal of AST is to provide a reliable predictor of how an organism is likely to respond to antimicrobial therapy in the infected host. The selection of a particular AST method is based on many factors such as validation data, practicality, flexibility, automation, cost, reproducibility, accuracy, and individual preference. In the modern methodologies, use of genotypic approaches for detection of antimicrobial resistance genes has also been promoted as a way to increase the speed and accuracy of susceptibility testing. Many DNA-based assays are being developed to detect bacterial antibiotic resistance at the genetic level. These methods, when used in conjunction with phenotypic analysis, offer the promise of increased sensitivity, specificity, and speed in the detection of specific known resistance genes and can be used in tandem with traditional laboratory AST methods. The invention of new molecular technologies in genomics and proteomics is shifting traditional techniques for bacterial classification, identification, and characterization in the twenty-first century toward methods based on the elucidation of specific gene sequences or molecular components of a cell. The new methods can

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be rapid, offer high throughput, and produce unprecedented levels of discrimination among strains of bacteria and archaea.

13.1 Introduction

The traditional as well as modern way for detecting and identifying bacteria from food, clinical, environment, or other samples is primarily based on culturing, enumeration, and isolation of pure colonies and further identification analysis using compact instrumentation and analytical techniques. However, tremendous increase in resistance among microbial pathogens is of utmost concern globally and is significantly recognized by the World Organisation for Animal Health (OIE), the Food and Agriculture Organization (FAO), and the World Health Organization (WHO) as a serious human and animal health problem throughout the world.

The phenomenon of development of bacterial antimicrobial resistance is neither an unexpected nor a new phenomenon. It is an increasingly troublesome situation because of the fact that new emerging resistance phenotypes are occurring among many bacterial pathogens and even commensal organisms worldwide. There are several approaches by which many infections could be treated successfully according to the past clinical experience (i.e., empirical therapy) (Walker 2007); however, resistance has been observed to different antimicrobial agents tapproved for use in human and veterinary clinical medicine. The combination of the variety of antimicrobial agents currently available makes the selection of an appropriate agent an increasingly more challenging task. This situation has made clinicians more dependent on data from in vitro antimicrobial susceptibility testing (ATS) and highlights the importance of the diagnostic laboratory in clinical practice. It is essential that AST methods provide reproducible results in day-to-day laboratory use and that the data be comparable with those results obtained by an acknowledged "gold standard" reference method. In the absence of standardized methods or reference procedures, susceptibility results from different laboratories cannot be reliably compared. The methods used to select samples for inclusion in antimicrobial resistance surveillance programs, as well as the methods used for primary bacterial isolation, are also important factors that should be standardized or harmonized to allow direct comparison of data between different regions; consideration of these issues is addressed in an OIE document (Dehaumont 2004). Furthermore, a greater understanding of the multiple factors that could affect the overall outcome of susceptibility testing has become clearer. National Committee for Clinical Laboratory Standards (NCCLS) provides various guidelines referring to the standardization and interpretive aspects for AST methodologies with the recent advances of modern molecular techniques such as genomics and proteomics.

It has also been notified that antimicrobial susceptibility along with the use of modern approaches such as genomic and proteomics has offered attractive

alternatives to conventional and advanced microbiological procedures for characterizing and identifying microorganisms. The discrimination and identification of bacteria within mixed natural populations is also a rapidly developing field that utilizes some of the same techniques. These new modern methods can provide a rapid, multidimensional data output with taxonomically relevant molecular information on both individual strains and whole populations (Liu and Stahl 2007; Logue et al. 2008).

Methods of bacterial identification can be broadly delimited into genotypic techniques based on profiling of an organism's genetic material (primarily its DNA) and phenotypic techniques based on profiling either an organism's metabolic attributes or some aspect of its chemical composition. Genotypic techniques have the advantage over phenotypic methods that:

- They are independent of the physiological state of an organism
- They are not influenced by the composition of the growth medium or by the organism's phase of growth

Phenotypic techniques instead of phenetic once can yield more direct functional information revealing the fate of metabolic activities taking place to aid the survival, growth, and development of the organism. These may further be embodied, in a microbe's adaptive ability to grow on a certain substrate, or in the degree to which it is resistant to a legion of antibiotics. Based upon the complementation between genotypic and phenotypic approaches, this chapter provides the following modern approaches for determining antimicrobial drug-resistant bacteria.

13.2 Antimicrobial Susceptibility Tests

Antimicrobial susceptibility testing (AST) are among the most prevalent methods to determine bacterial susceptibility to various antimicrobials available. Among several approaches, selection of a particular method is based on some of the responsible factors such as:

- Practicality
- Flexibility
- Automation
- Cost
- Reproducibility
- Accuracy
- Individual preference

Based upon these parameters, following three methods have been shown to consistently provide reproducible and repeatable results when followed correctly (Clinical and Laboratory Standards Institute (CLSI) 2008; Walker 2007):

(i) Disk diffusion including agar well diffusion assay,
(ii) Broth dilution including micro- and macro-broth dilution,
(iii) Agar dilution.

13.3 Disk Diffusion Method

This method refers to the diffusion of antimicrobial agent/antibiotics of a specified concentration from disks, tablets, or strips and even through wells into the solid culture medium that has been seeded with the selected inoculum isolated in a pure culture. Disk diffusion is based on the determination of an inhibition zone proportional to the bacterial susceptibility to the antimicrobial present in the disk, strip, and well. The diffusion of the antimicrobial agent into the seeded culture media results in the formation of a concentration gradient of the antimicrobial through the agar gel. When the concentration of the antimicrobial becomes so diluted that it can no longer inhibit the growth of the test bacterium, the zone of inhibition is demarcated. The diameter of this zone of inhibition around the antimicrobial disk is related to minimum inhibitory concentration (MIC) for that particular bacterium/antimicrobial combination.

The zone of inhibition correlates inversely with the MIC of the test bacterium. Generally, the larger the zone of inhibition, the lower the concentration of antimicrobial required to inhibit the growth of the organisms. However, this depends on the concentration of antibiotic in the disk and its diffusibility. It is also to be noted that disk diffusion tests based solely on the presence or absence of a zone of inhibition without regard to the size of the zone of inhibition are not acceptable AST methodology.

13.4 Considerations for the Use of The Disk Diffusion Methodology

Disk diffusion method for determining antimicrobial susceptibility is very straightforward to perform, is reproducible, and does not require expensive equipment.

The major advantages of diffusion methods are:

(a) Low cost of method
(b) Ease in modifying test antimicrobial agents when required
(c) This can be used as a screening test against large numbers of isolates
(d) This can identify a subset of isolates for further testing by other methods, such as determination of MICs

Manual measurement of zones of inhibition may be time-consuming and error prone; therefore, recently developed automated zone-reading devices are available that can be integrated with laboratory reporting and data-handling systems. Disks should also be distributed evenly so that the zones of inhibition around

antimicrobial disks in the disk diffusion test do not overlap to such a degree that the zone of inhibition cannot be determined. Generally, this can be accomplished if the disks are no closer than 24 mm from center to center, though this is dependent on disk concentration and the ability of the antimicrobial to diffuse in agar.

13.5 Broth and Agar Dilution Methods

The major aim of these dilution methods is to determine the lowest concentration of the assayed antimicrobial that inhibits the visible growth of the bacterium being tested, i.e., minimum inhibitory concentration (MIC, usually expressed in μg/ml or mg/liter). However, the MIC does not always represent an absolute value. The "true" MIC is a point between the lowest test concentration that inhibits the growth of the bacterium and the next lower test concentration. Therefore, MIC determinations performed using a dilution series may be considered to have an inherent variation of one dilution.

Antimicrobial ranges should encompass both the interpretive criteria (susceptible, intermediate, and resistant) for a specific bacterium/antibiotic combination and appropriate quality control reference organisms. Dilution methods, practically, appear to be more reproducible and quantitative than agar disk diffusion. However, antibiotics are usually tested in doubling dilutions, which can produce inexact MIC data. Any laboratory that intends to use a dilution method and set up its own reagents and antibiotic dilutions should have the ability to obtain, prepare, and maintain appropriate stock solutions of reagent-grade antimicrobials and to generate working dilutions on a regular basis. It is then essential that such laboratories should use quality control organisms to assure accuracy and standardization of their procedures.

13.6 Broth Dilution

Broth dilution is a technique in which the concentration of a given bacterium (Test Bacteria) is predetermined by densitometer and optimal or appropriate concentration of respective bacterium is tested against varying concentrations of an antimicrobial agent (usually serial twofold dilutions) in a liquid medium of predetermined, documented formulation. The broth dilution method can be performed either in tubes containing a minimum volume of 2 ml (macrodilution) or in smaller volumes using microtitration plates (microdilution) or 96-well plate method. Numerous microtiter plates containing lyophilized prediluted antibiotics within the wells are also commercially available. The use of identical lots in microdilution plates may assist in the minimization of variation that may arise due to the preparation and dilution of the antimicrobials from different laboratories. However, use of these plates, with a documented test protocol, including

specification of appropriate reference organisms, will facilitate the comparability of results among laboratories.

Due to the fact that most broth microdilution antimicrobial test panels are prepared commercially, this method is less flexible than agar dilution or disk diffusion in adjusting to the changing needs of the surveillance/monitoring program. Because the purchase of antimicrobial plates and associated equipment may be costly, this methodology may not be feasible for some laboratories as well.

13.7 Agar Dilution

Agar dilution methods involves the incorporation of varying concentrations of antimicrobial agent into an agar medium (such as Muller Hilton Agar, Nutrient Agar, Blood Agar, etc.) usually using serial twofold dilutions, followed by the application of a defined bacterial inoculum to the agar surface of the plate. These results are often considered as the most reliable for the determination of an MIC for the test bacterium/antimicrobial combination. Agar dilution is often recommended as a standardized AST method for fastidious organisms (CLSI 2006), such as anaerobes and *Helicobacter* species.

The advantages of agar dilution methods include:

(a) The ability to test multiple bacteria, except bacteria that swarm, on the same set of agar plates at the same time
(b) The potential to improve the identification of MIC end points and extend the antibiotic concentration range
(c) The possibility to semi-automate the method using an inoculum-replicating apparatus. Commercially produced inoculum replicators are available and these can transfer between 32 and 60 different bacterial inoculum to each agar plate

However, agar dilution methods also have certain disadvantages which are as follows:

(a) Although automated method also available, but if not automated, they are very laborious and require substantial economic and technical resources,
(b) After preparation of agar plates, they normally should be used within a week (or less, depending on the antimicrobials tested),
(c) End points are neither easy to read nor is the purity of the inoculum easy to verify.

13.8 Other Bacterial AST and Specific Antimicrobial Resistance Tests

Commercially available gradient strips can also be used to determine bacterial antimicrobial MICs by using the diffusion phenomenon of a predetermined antibiotic concentration. However, the use of gradient strips can be very expensive and MIC discrepancies can be found when testing certain bacteria/antimicrobial combinations compared with agar dilution results (Ge et al. 2002; Rathe et al. 2009). Regardless of the AST method used, the procedures should be documented in detail to ensure accurate and reproducible results, and appropriate reference organisms should always be tested every time AST is performed in order to ensure accuracy and validity of the data.

Growth characteristics of the test bacterium significantly affect the choice of an appropriate AST system. In special circumstances, novel test methods and assays may be more appropriate for detection of particular resistance phenotypes. For example, chromogenic cephalosporin-based tests (e.g., nitrocefin) may provide more reliable and rapid results for beta-lactamase determination in certain bacteria (CLSI 2008), whereas inducible clindamycin resistance in *Staphylococcus* spp. may be detected using a disk diffusion method employing standard erythromycin and clindamycin disks in adjacent positions and measuring the resultant zones of inhibition (e.g., D-zone or D-test) (Zelazny et al. 2005).

Similarly, extended-spectrum beta-lactamase (ESBL) activity in certain bacteria can also be detected by using standard disk diffusion susceptibility test methods incorporating specific cephalosporins (cefotaxime and ceftazidime) in combination with a beta-lactamase inhibitor (clavulanic acid) and measuring the resulting zones of inhibition (CLSI 2008). Also penicillin-binding protein 2a (PBP 2a) can be detected in methicillin-resistant staphylococci with a latex agglutination test (Stepanovic et al. 2006). It is essential that testing of known positive and negative control strains occurs alongside clinical isolates to ensure accurate results.

13.9 Future Directions in Antimicrobial Susceptibility/Resistance Detection

Modern science uses the genotypic approaches for detection of antimicrobial resistance genes in order to increase the rapidity and accuracy of susceptibility testing (Cai et al. 2003; Chen et al. 2005). Numerous DNA-based assays are being developed to detect bacterial antibiotic resistance at the genetic level. The newest and perhaps most state-of-the-art approach is to predict antimicrobial resistance phenotypes *via* identification and characterization of the known genes that encode specific resistance mechanisms.

Methods that employ the use of comparative genomics, genetic probes, microarrays, nucleic acid amplification techniques (e.g., polymerase chain

reaction), and DNA sequencing offer the promise of increased sensitivity, specificity, and speed in the detection of specific known resistance genes (Cai et al. 2003; Chen et al. 2005; Perreten et al. 2005). Genotypic methods have been successfully applied to supplement traditional AST phenotypic methods for other organisms including methicillin-resistant staphylococci, vancomycin-resistant enterococci, and detection of fluoroquinolone resistance mutations (Cai et al. 2003; Chen et al. 2005; Perreten et al. 2005).

13.9.1 Genotypic Methods

There are two broad categories of genotypic microbial identification methods:

(a) Genotypic methods based on pattern or fingerprint techniques
(b) Methods of sequence-based techniques

Pattern-based or fingerprinting techniques typically employ a systematic method to produce a series of fragments from a target organism's chromosomal DNA. These fragments are then separated by size to generate a profile, or fingerprint, which is unique to that organism and its very close relatives. Using this DNA profile information, researchers can create a library, or database, of fingerprints from known organisms, to which test organisms can be compared. When the profiles of two organisms match, they can be considered very closely related, usually at the strain or species level.

Sequence-based techniques are based on determining the sequence of a specific part of DNA strand, which is usually, but not always, associated with a specific gene. In general, the approach is the same as for genotyping: a database of specific target DNA sequences is generated, and then a test sequence is compared with it. The degree of similarity, or match, between the two sequences is a measurement of how closely related the two organisms are to one another. A number of computer algorithms have been created by researchers that can compare multiple sequences to one another and build a phylogenetic tree based on the results (Ludwig and Klenk 2001).

Both fingerprinting techniques and sequence-based methods have strengths but weaknesses too. The sequence-based methods, for example, analysis of the 16S rRNA gene, have proved more effective in providing broader phylogenetic relationships among bacteria at the genus, family, order, and phylum levels, while fingerprinting-based methods are good at distinguishing strain- or species-level relationships but are less reliable for providing relatedness above the species or genus level (Vandamme et al. 1996). Specific genotyping methodologies for the identification of bacteria may utilize a variety of different fingerprinting- or sequence-based methods, either alone or, more often, in combination. These techniques provide both greater accuracy for identification and higher sample throughput. Examples of some of the most widely used techniques are the following.

13.9.2 Fingerprinting-Based Methodologies

These techniques are the most frequently used genotypic methods for the identification and characterization of new strain of bacteria (Versalovic et al. 1994; Cocconcelli et al. 1995; Vos et al. 1995; Lin et al. 1996). The most widely used methods are as below:

(a) Repetitive element PCR (rep-PCR)
(b) Amplified fragment length polymorphism (AFLP)
(c) Random amplification of polymorphic DNA (RAPD)

13.9.3 Sequence-Based Methodologies

Sequence-based methods provide important information for bacterial identification.

13.9.4 Multi-locus Sequence Based Methods

Multi-locus sequencing is one of the newest and most powerful methods which is developed to identify microbial species. According to the principle, this technique is akin to 16S rRNA gene sequence comparisons, except that, instead of one gene, the fragments of multiple "housekeeping" genes are each sequenced, and the combined sequences are put together, or concatenated, into one long sequence that can be compared with other sequences. The housekeeping genes generally encode proteins that carry out essential cellular processes, for example, the gyrase B subunit (*gyrB*), the alpha and beta subunits of RNA polymerase (*rpoA* and *rpoB*), and *recA*, a gene encoding for an enzyme important in DNA repair; there are a host of others (Zeigler 2003). Loci for housekeeping gene are present in most of the cells and tend to be conserved among different organisms. As a result, general-purpose primers can be designed that will work using PCR to amplify the same genes across multiple genera; however, in many cases, truly universal primer sets are not possible, so primers need to be designed for specific families or orders of bacteria.

Currently used multi-locus sequencing is two multi-locus sequencing strategies, i.e., Multi-Locus Sequence Typing (MLST) and Multi-Locus Sequence Analysis (MLSA). MLST is a well-defined approach that uses a suite of 6–10 genetic loci, with appropriate primers for each locus to allow PCR amplification and sequencing of the products (usually 400–600 base pairs) (Maiden et al. 1998). MLSA also involves sequencing of multiple fragments of conserved protein encoding genes, but it uses a more ad hoc approach to choosing the genes for comparative analysis. A smaller subset ($\leq$6) of genes or loci is typically used in MLSA than is used in MLST (Gevers et al. 2005).

13.9.5 Microarrays

Microarrays are among the highly imperative technologies that simultaneously identify specific microbes and provide ecological context for the population structure and functional structure of a given microbial community. Microarray is based on spotting probes for hundreds or thousands of genes onto a substrate and then hybridizing sample DNA or RNA to it. The sample DNA or RNA is labeled with a fluorescent reporter molecule so that samples that hybridize with probes on the microarray can be detected rapidly. For bacterial identification, several recapitulations of a "phylochip" that utilizes the small subunit of ribosomal gene as a target have been developed, both for specific and for very broad groups of environmental bacteria (Liu et al. 2001; Wilson et al. 2002). Geochip is another example, which has been developed to identify microbes involved in essential biogeochemical processes such as metal transformations, contaminant degradation, and primary carbon cycling (He et al. 2007). Microarrays are moving forward rapidly, both for diagnostic purposes and for understanding the fundamentals of disease pathology (Frye et al. 2006; Richter et al. 2006). However, because of their inherent complexity and relative expense, microarrays have yet to be used as standard methods in microbial identification.

13.9.6 Proteomics in Bacterial Identification and Characterization

Use of proteomics in conjunction with mass spectrometry allows rapid interrogation of biomolecules produced by an organism and offers an excellent complement to classical microbiological and genomics-based techniques for bacterial classification, identification, and phenotypic characterization. The most extensively used proteomic technologies for bacterial identification and characterization include Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF-MS); Electro-Spray Ionization Mass Spectrometry (ESI-MS); Surface-Enhanced Laser Desorption/Ionization (SELDI) Mass Spectrometry; One- or Two-Dimensional Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis (SDS-PAGE); or the combination of Mass Spectrometry, Gel Electrophoresis, and Bioinformatics. Fourier-Transform Infrared Spectroscopy (FT-IR) has also been used extensively to classify and identify bacterial samples (Al-Qadiri et al. 2006). Figure 13.1 illustrates a generalized view of integrated proteomics flowchart.

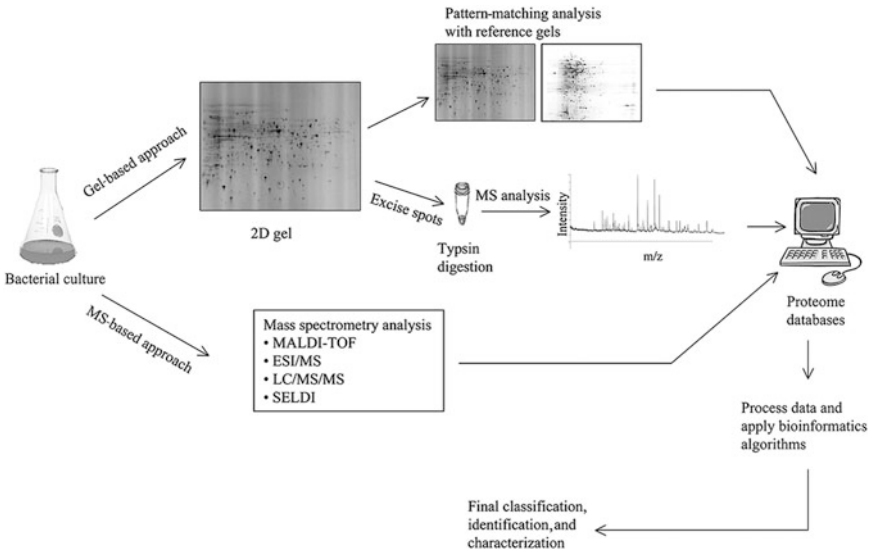

Fig. 13.1 Overview of proteomics approaches in bacterial identification and characterization. The bacterial sample can be analyzed using either a gel-based or a mass spectrometry (MS)-based approach. In the gel-based approach, bacterial lysate is prepared and run on one- or two-dimensional sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). The SDS-PAGE can be analyzed by comparing it directly with available gel images in the database or by excising the protein spots and using trypsin digestion and mass analysis for identification. On the basis of the protein pattern analysis or the identified proteins, or both, the bacterium from which the lysate was prepared will be identified using bioinformatics analysis (database search and computer algorithm analysis). In the MS-based approach, bacterial lysates or the whole cell are analyzed using different mass spectrometry techniques, such as matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF), electrospray ionization mass spectrometry (ESI-MS), liquid chromatography tandem mass spectrometry (LC/MS/MS), and surface-enhanced laser desorption ionization mass spectrometry (SELDI). The unknown bacterium will be identified either by comparing the resulting mass spectra with a collective proteomics database containing mass spectra of known bacteria or by searching and matching the sequence of a panel of proteins with proteins of known bacteria in the protein database

13.9.7 Mass Spectrometry

Mass spectrometry is a most powerful analytical tool that has been used to identify unknown compounds, quantify known compounds, and elucidate the structure and chemical properties of molecules. Soft Ionization Methods such as MALDI-TOF-MS and ESI-MS for the analysis of biological molecules are a major breakthrough in mass spectrometry. Before the development of the soft ionization method, the application of mass spectrometry to biological materials was limited by the requirement that the sample be in vapor phase before ionization. Soft ionization has made it possible to study larger biological molecules and perform analyte sampling and ionization directly from native samples, including whole cells, using mass

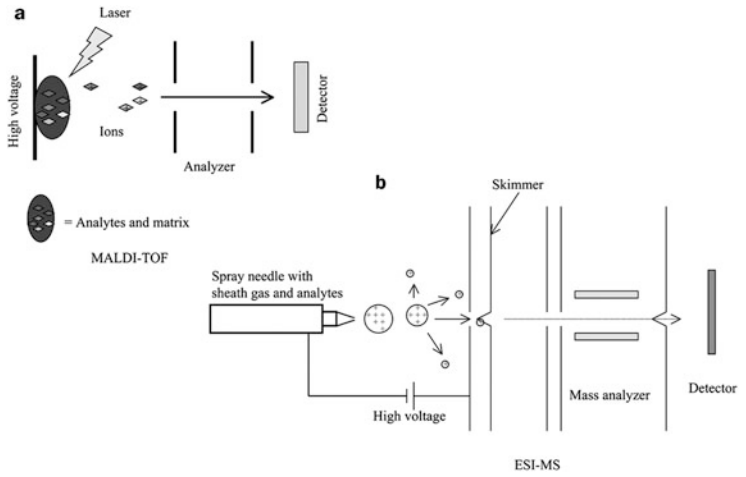

Fig. 13.2 Schematic representation of soft ionization techniques used in mass spectrometry. (**a**) Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry. The sample to be analyzed is mixed with organic matrices and deposited on the sample plate in the form of a small spot. The mixture is ionized by the laser beam. The resulting ions move toward the mass analyzer, and the mass is detected to obtain the mass spectrum. (**b**) Electrospray ionization mass spectrometry (ESI-MS). The analyte is mixed with a solvent and sprayed from a narrow tube. Positively charged droplets in the spray move toward the mass spectrometer sampling orifice under the influence of electrostatic forces and pressure differentials. As the droplets move to the orifice, the solvent evaporates, causing the analyte ions to move toward the analyzer for mass analysis

spectrometry (Fenn et al. 1989). Figure 13.2 illustrates a simplified schematic representation of MS.

13.9.8 Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry

MALDI-TOF-MS is another precious mass spectral method for bacterial analysis (Lay 2001; Dare 2006). It is highly significant because of the following facts:

(a) This tool can be used to analyze whole bacterial cells directly.
(b) It can produce relatively simple, reproducible spectra patterns over a broad mass range under well-controlled experimental conditions.
(c) The spectra patterns contain characteristic information that can be used to identify and characterize bacterial species by comparing the spectra fingerprints of the unknown species with known library fingerprints.
(d) A number of known, taxonomically important protein markers can be used directly for identifying bacterial species.

MALDI-TOF-MS is rapidly becoming an accepted technology for bacterial identification either by comparison with archived reference spectra or by co-analysis with cultures of known bacteria. A variety of bacteria have been analyzed using MALDI-TOF-MS, including *Staphylococcus* species (Edwards-Jones et al. 2000), *Mycobacteria* species (Pignone et al. 2006), and extremophilic bacteria and archaea (Krader and Emerson 2004). The most prevalent example of its use is *Staphylococcus aureus*, a bacterium commonly found on human skin, which causes infection during times of uncontrolled growth. Improper use of antibiotics has rendered *S. aureus* resistant to the methicillin class of antibiotics (Edwards-Jones et al. 2000). The first outbreak of methicillin-resistant *S. aureus* (MRSA) was recorded in a European hospital in the early 1960s. Since then, the threat of MRSA has spread from hospitals and clinical settings to schools and public communities, thus necessitating the use of techniques that can rapidly identify and discriminate MRSA from methicillin-sensitive *S. aureus* (MSSA). Edwards-Jones et al. (2000) developed a MALDI-TOF-MS method for the identification, typing, and discrimination of MRSA and MSSA. In this method, a sample is taken from a single bacterial colony and smeared onto a sample slide. The appropriate matrix is applied to the sample, which is then analyzed using MALDI-TOF-MS which shows that MRSA and MSSA yield distinct spectral peaks that allow for rapid distinction between the two and, therefore, hypothetically, for appropriate treatment of *S. aureus* infections with respect to their resistance to antibiotics. Bruker Daltonics' MALDI BioTyper is a MALDI-TOF-MS system which is based on the measurement of high-abundance proteins, including many ribosomal proteins in a microorganism (Mellmann et al. 2008). The system is equipped with bioinformatics tools (clustering and phylogenetic dendrogram construction) that allow for the rapid identification and characterization of a known or unknown bacterial culture on the basis of proteomics signatures (Lay 2001; Dare 2006).

13.9.9 Electrospray Ionization Mass Spectrometry

ESI-MS is also an important tool for bacterial identification and characterization, especially for the analysis of cellular components. In this methodology, proteins expressed by the bacteria can be extracted from the lysed cells and analyzed using ESI-MS. This technique allows for the analysis of both intracellular and extracellular proteins, carbohydrates, and lipids. ESI-MS also has the advantage in its ability to perform tandem mass spectrometry during which the protein of interest can be fragmented for a second mass analysis. It further provides protein fragment sequence information or a peptide fragmentation fingerprint which would be helpful in database search to identify that specific protein. Due to this technique, accuracy of protein identification is significantly increased as compared with identification using only molecular weight information from a single MALDI-TOF-MS analysis.

Krishnamurthy and Ross (1996) reported that the total analysis time leading to unambiguous bacterial identification in samples is less than 10 min, with reproducible results. Recent advances in this technology made it possible to identify and characterize bacterial strains rapidly and effectively using a combination of PCR and ESI-MS technology (Sampath et al. 2007). Combination of MALDI-TOF-MS with MLST analysis is also developed recently in order to analyze nucleic acids in a pathogenic bacterial model, i.e., *Neisseria meningitidis* (Honisch et al. 2007). Integrated genotypic and proteomics technologies provide very powerful tool for bacterial identification and characterization (Bons et al. 2005).

13.10 Surface-Enhanced Laser Desorption/Ionization

SELDI is a relatively new and advanced technology which is specifically designed to perform mass spectrometric analysis of protein mixtures retained on chemically (e.g., cationic, ionic, hydrophobic) or biologically (e.g., antibody, ligand) modified chromatographic chip surfaces. These varied chemical and biochemical surfaces allow differential capture of proteins based on the intrinsic properties of the proteins themselves. The SELDI mass spectrometer produces spectra of complex protein mixtures based on the mass-to-charge ratio of the proteins in the mixture and their binding affinity to the chip surface. Differentially expressed proteins may then be determined from these protein profiles by comparing peak intensity. Figure 13.3 illustrates the general procedure of SELDI.

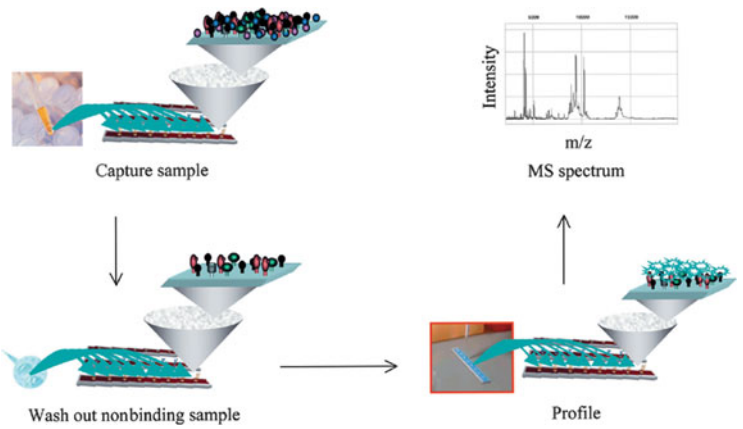

Fig. 13.3 Schematic representation of the surface-enhanced laser desorption/ionization technique (abbreviations: *MS* mass spectrometry, *m/z* mass-to-charge ratio). This technique utilizes aluminum-based chips, engineered with chemically or biologically modified surfaces. These varied surfaces allow the differential capture of proteins based on the intrinsic properties of the proteins themselves. Bacterial lysates are applied directly to the surfaces, where proteins with affinities to the surface will bind. Following a series of washes to remove nonspecifically bound proteins, the bound proteins are profiled using the integrated mass analyzer to generate a mass spectrum for further analysis.

Applicability of SELDI for detecting and identifying bacterial pathogens and virulence factors has been extensively studied (Seo et al. 2004). These studies demonstrate the alternative approach of SELDI technology to other existing techniques for exploring bacterial proteomes and ultimately permitting bacterial identification based on a comparison of protein profiles and patterns. Lundquist et al. (2005) showed that SELDI-TOF-MS is capable of generating unique and reproducible protein profiles for each subspecies, allowing the subspecies to be distinguished from one another. Using this technology, four subspecies of *Francisella tularensis*, causative agent of tularemia in humans, have been explored. It has been also observed that out of the four subspecies of *F. tularensis*, *tularensis* is highly infectious.

13.11 Gel-Based Bacterial Characterization and Identification

Bacteria may also be differentiated on the basis of their cellular protein contents. The most established technique for examining cellular protein content is to lyse cells and separate their entire protein complement using SDS-PAGE. This results in a migration pattern of the protein bands that is characteristic for a given bacterial strain (Vandamme et al. 1996). Researchers can identify bacteria by comparing their migration patterns with reference gel patterns in an established database. However, because SDS-PAGE analysis is slow and labor intensive, and because the application necessitates precise culture conditions that yield fairly large amounts of sample material, it is not particularly useful for rapid identification of bacteria, particularly for field and point-of-care applications.

Two-dimensional gel electrophoresis (2DE), i.e., the combination of isoelectric focusing (IEF) and SDS-PAGE, affords a high-resolution separation of up to several thousand spots in a single gel analysis. In 1975, O'Farrell (1975) introduced 2DE as a method for separating complex mixtures of cellular proteins. In 2DE, proteins are separated by IEF electrophoresis in a pH gradient according to each protein's isoelectric point in the first dimension, followed by the second-dimension SDS-PAGE separation according to the relative molecular weight of each protein. After the second-dimension separation, the gel can be stained with standard or sensitive staining solutions so that protein spots can be visualized and analyzed. Protein gel patterns or 2DE maps from known bacteria can be further scanned, analyzed, and stored in a reference database. To identify an unknown species, a 2DE map from the unknown sample is generated by running a 2DE gel and then comparing it with 2DE maps in the reference database for identification.

When used as a stand-alone technique, 2DE is most often used for analyzing protein mixtures, isolating proteins of interest for identification, and comparing differential expression patterns of different types of samples. For more complex

proteomics analysis, 2DE is greatly enhanced when combined with mass spectrometry. Redmond et al. (2004) analyzed the exosporium of *Bacillus anthracis* spores by isolating the proteins from the outer casing of the spore using SDS-PAGE and analyzing the isolated protein using delayed-extraction MALDI-TOF-MS. The team identified several proteins associated with the exosporium of *B. anthracis*. Using these methods, the whole proteome or sub-proteome has also been made available for many other bacteria, including *E. coli, Bacillus subtilis*, *S. aureus*, *Pseudomonas aeruginosa*, and *Helicobacter pylori* (Nouwens et al. 2000; Hecker et al. 2003; Peng et al. 2005; Pieper et al. 2006). A collective proteomics database with complete 2DE maps and mass spectra of known bacteria will allow investigators to compare and identify unknown bacteria.

PCR methods have been described for betalactamases, aminoglycoside-inactivating enzymes, and tetracycline efflux genes (Cai et al. 2003; Chen et al. 2005; Frye et al. 2010; Perreten et al. 2005). Technological innovations in DNA-based diagnostics should allow for the detection of multiple resistance genes and/or variants during the same test. The development of rapid diagnostic identification methods and genotypic resistance testing should help reduce the emergence of antimicrobial resistance, by enabling the use of the most appropriate antimicrobial when therapy is initiated. However, DNA techniques have to be demonstrated to be complementary to AST methods and results. Additionally, new technological advances may facilitate the ability to probe bacterial species for large numbers of antimicrobial resistance genes quickly and cheaply, thereby providing additional relevant data for surveillance and monitoring programs (Frye et al. 2010). However, despite the new influx of genotypic tests, documented and agreed upon phenotypic AST methods will still be required in the near future to detect emerging resistance mechanisms among bacterial pathogens.

13.12 Conclusion

Emergence of antibiotic resistance among microbial pathogens is a major concern throughout the world. This situation is aggressively worsened due to the wide spreading of resistance genes by horizontal gene transfer, ultimately leading to the novel resistant strains termed as multidrug-resistant (MDR), extremely drug-resistant (XDR), and total drug-resistant (TDR) bacterial pathogens. Although a variety of methods exist for evaluating the resistance patterns, the major goal of in vitro antimicrobial susceptibility testing remains to provide a reliable predictor of how a microorganism is likely to respond to antimicrobial therapy in the infected host. This type of information aids the clinician in selecting the appropriate antimicrobial agent, provides data for surveillance, and aids in developing antimicrobial judicious use policies (World Organization for Animal Health 2010). In vitro antimicrobial susceptibility testing can be performed using a variety of formats, the most common being disk diffusion, agar dilution, broth macrodilution, broth microdilution, and a concentration gradient test. Each of these procedures

requires the use of specific testing conditions and methods, including media, incubation conditions and times, and the identification of appropriate quality control organisms along with their specific parameters. It is essential that AST methods provide reproducible results in day-to-day laboratory use and that the data be comparable with those results obtained by an acknowledged "gold standard" reference method. In the absence of standardized methods or reference procedures, antimicrobial susceptibility/resistance results from different laboratories cannot be reliably compared.

The use of genotypic approaches for detection of antimicrobial resistance genes has also been promoted as a way to increase the rapidity and accuracy of susceptibility testing. Additionally, new technological advances in molecular techniques (e.g., microarray) may facilitate the ability to probe bacterial species for large numbers of antimicrobial resistance genes quickly and cheaply, thereby providing additional relevant data into surveillance and monitoring programs (Ojha and Kostrzynska 2008; Poxton 2005). Despite the new influx of genotypic tests, however, standardized phenotypic AST methods will still be required in the near future to detect emerging resistance mechanisms among bacterial pathogens.

Advanced genomics and proteomics technologies play a critical role in bacterial identification and characterization and have a number of practical applications, aside from being fundamental to questions of bacterial systematics, taxonomy, and evolution. Rapid identification and discrimination of pathogenic microbes has a major impact on public health in terms of correct diagnosis and timely disease treatment. This kind of information has a significant impact for treatment options. The ability to rapidly identify these individual organisms within populations of thousands of different species is essential for understanding how they will affect our ecosystems. Bacterial characterization will also assist in elucidating the mechanisms that govern microbial pathogenesis and allow for the discovery of important protein targets essential to the development of vaccines, diagnostic kits, and therapeutics for infectious diseases. It is these kinds of applications that make the continued development of techniques for bacterial identification important both for basic science and for the maintenance of human and environmental health.

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References

Al-Qadiri HM, Lin M, Cavinato AG, Rasco BA (2006) Fourier transform infrared spectroscopy, detection and identification of *Escherichia coli* O157:H7 and *Alicyclobacillus* strains in apple juice. Int J Food Microbiol 111:73–80

Bons JA, Wodzig WK, van Dieijen-Visser MP (2005) Protein profiling as a diagnostic tool in clinical chemistry: a review. Clin Chem Lab Med 43:1281–1290

Cai HY, Archambault M, Gyles CL, Prescott JF (2003) Molecular genetic methods in the veterinary clinical bacteriology laboratory: current usage and future applications. Anim Health Rev 4:73–93

Chen S, Zhao S, Mcdermott PF, Schroeder CM, White DG, Meng J (2005) A DNA microarray for identification of virulence and antimicrobial resistance genes in *Salmonella* serovars and *Escherichia coli*. Mol Cell Probes 19:195–201

Clinical and Laboratory Standards Institute (CLSI) (2006) Document M45-A. Methods for antimicrobial dilution and disk susceptibility of infrequently isolated or fastidious bacteria; approved guideline. CLSI, Wayne, PA

Clinical and Laboratory Standards Institute (CLSI) (2008) Document M31-A3. Performance standards for antimicrobial disk and dilution susceptibility tests for bacteria isolated from animals, approved standard, third edition. CLSI, Wayne, PA

Cocconcelli PS, Porro D, Galandini S, Senini L (1995) Development of RAPD protocol for typing of strains of lactic-acid bacteria and enterococci. Lett Appl Microbiol 21:376–379

Dare D (2006) Rapid bacterial characterization and identification by MALDI-TOF mass spectrometry. In: Tang Y-W, Stratton CW (eds) Advanced techniques in diagnostic microbiology. Springer, New York, pp 117–133

Dehaumont P (2004) OIE international standards on antimicrobial resistance. J Vet Med [Ser B] 51:411–414

Edwards-Jones V, Claydon MA, Evason DJ, Walker J, Fox AJ, Gordon DB (2000) Rapid discrimination between methicillin-sensitive and methicillin-resistant *Staphylococcus aureus* by intact cell mass spectrometry. J Med Microbiol 49:295–300

Fenn JB, Mann M, Meng CK, Wong SF, Whitehouse CM (1989) Electrospray ionization for mass spectrometry of large biomolecules. Science 246:64–71

Frye JG, Jesse T, Long F, Rondeau G, Porwollik S, McClelland M, Jackson CR, Englen M, Fedorka-Cray PJ (2006) DNA microarray detection of antimicrobial resistance genes in diverse bacteria. Int J Antimicrob Agents 27:138–151

Frye JG, Lindsey RL, Rondeau G, Porwollik S, Long G, Mcclelland M, Jackson CR, Englen MD, Meinersmann RJ, Berrang ME, Davis JA, Barrett JB, Turpin JB, Thitaram SN, Fedorka-Cray PJ (2010) Development of a DNA microarray to detect antimicrobial resistance genes identified in the National Center for Biotechnology Information Database. Microb Drug Resist 16:9–19

Ge B, Bodeis S, Walker RD, White DG, Zhao S, Mcdermott PF, Meng J (2002) Comparison of Etest and agar dilution for in vitro antimicrobial susceptibility testing of *Campylobacter*. J Antimicrob Chemother 50:487–494

Gevers D, Cohan FM, Lawrence JG, Spratt BG, Coenye T, Feil EJ, Stackebrandt E, Van de Peer Y, Vandamme P et al (2005) Re-evaluating prokaryotic species. Nat Rev Microbiol 3:733–739

He Z, Gentry TJ, Schadt CW, Wu L, Liebich J, Chong SC et al (2007) GeoChip: a comprehensive microarray for investigating biogeochemical, ecological and environmental processes. ISME J 1:67–77

Hecker M, Engelmann S, Cordwell SJ (2003) Proteomics of *Staphylococcus aureus*—current state and future challenges. J Chromatogr B Analyt Technol Biomed Life Sci 787:179–195

Honisch C, Chen Y, Mortimer C, Arnold C, Schmidt O, van den Boom D, Cantor CR, Shah HN, Gharbia SE (2007) Automated comparative sequence analysis by base-specific cleavage and mass spectrometry for nucleic acid-based microbial typing. Proc Natl Acad Sci 104:10649–10654

Krader P, Emerson D (2004) Characterization of Archaea and some extremophilic bacteria using matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry. Extremophiles 8:259–268

Krishnamurthy T, Ross PL (1996) Rapid identification of bacteria by direct matrix-assisted laser desorption/ionization mass spectrometric analysis of whole cells. Rapid Commun Mass Spectrom 10:1992–1996

Lay JO Jr (2001) MALDI-TOF mass spectrometry of bacteria. Mass Spectrom Rev 20:172–194

Lin JJ, Kuo J, Ma J (1996) A PCR-based DNA fingerprinting technique: AFLP for molecular typing of bacteria. Nucleic Acids Res 24:3649–3650

Liu WT, Stahl DA (2007) Molecular approaches for the measurement of density, diversity, and phylogeny. In: Hurst CJ, Crawford RL, Garland JL, Lipson DA, Mills AL, Stetzenbach LD (eds) Manual of environmental microbiology, 3 edn. ASM Press, Washington, DC, pp 139–156

Liu WT, Mirzabekov AD, Stahl DA (2001) Optimization of an oligonucleotide microchip for microbial identification studies: a non-equilibrium dissociation approach. Environ Microbiol 3:619–629

Logue JB, Bürgmann H, Robinson CT (2008) Progress in the ecological genetics and biodiversity of freshwater bacteria. BioScience 58:103–113

Ludwig W, Klenk H-P (2001) Overview: a phylogenetic backbone and taxonomic framework for prokaryotic systematics. In: Boone DR, Castenholtz RW (eds) Bergey's manual of systematic bacteriology. Springer, Berlin, pp 49–65

Lundquist M, Caspersen MB, Wikström P, Forsman M (2005) Discrimination of *Francisella tularensis* subspecies using surface enhanced laser desorption ionization mass spectrometry and multivariate data analysis. FEMS Microbiol Lett 243:303–310

Maiden MCJ, Bygraves JA, Feil E, Morelli G, Russell JE, Urwin R, Zhang Q, Zhou J, Zurth K, Caugant DA, Feavers IM, Achtman M, Spratt BG (1998) Multilocus sequence typing: a portable approach to the identification of clones within populations of pathogenic microorganisms. Proc Natl Acad Sci USA 95:3140–3145

Mellmann A, Cloud J, Maier T et al (2008) Evaluation of matrix-assisted laser desorption ionization-time-of-flight mass spectrometry in comparison to 16S rRNA gene sequencing for species identification of nonfermenting bacteria. J Clin Microbiol 46:1946–1954

Nouwens AS, Cordwell SJ, Larsen MR, Molloy MP, Gillings M, Willcox MD, Walsh BJ (2000) Complementing genomics with proteomics: the membrane subproteome of *Pseudomonas aeruginosa* PAO1. Electrophoresis 21:3797–3809

O'Farrell PH (1975) High resolution two-dimensional electrophoresis of proteins. J Biol Chem 250:4007–4021

Ojha S, Kostrzynska M (2008) Examination of animal and zoonotic pathogens using microarrays. Vet Res 39:4–26

Peng X, Xu C, Ren H, Lin X, Wu L, Wang S (2005) Proteomic analysis of the sarcosine-insoluble outer membrane fraction of *Pseudomonas aeruginosa* responding to ampicilin, kanamycin, and tetracycline resistance. J Proteome Res 4:2257–2265

Perreten V, Vorlet-Fawer L, Slickers P, Ehricht R, Kuhnert P, Frey J (2005) Microarray-based detection of 90 antibiotic resistance genes of gram-positive bacteria. J Clin Microbiol 43:2291–2302

Pieper R, Gatlin-Bunai CL, Mongodin EF, Parmar PP, Huang ST, Clark DJ, Fleischmann RD, Gill SR, Peterson SN (2006) Comparative proteomic analysis of *Staphylococcus aureus* strains with differences in resistance to the cell wall-targeting antibiotic vancomycin. Proteomics 6:4246–4258

Pignone M, Greth K, Cooper J, Emerson D, Tang J (2006) Identification of mycobacteria by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. J Clin Microbiol 44:1963–1970

Poxton IR (2005) Molecular techniques in the diagnosis and management of infectious diseases: do they have a role in bacteriology? Med Princ Pract 14:20–26

Rathe M, Kristensen L, Ellermann-Eriksen S, Thomsen MK, Schumacher H (2009) Vancomycin-resistant *Enterococcus* spp.: validation of susceptibility testing and in vitro activity of vancomycin, linezolid, tigecycline and daptomycin. APMIS 118:66–73

Redmond C, Baillie LW, Hibbs S, Moir AJ, Moir A (2004) Identification of proteins in the exosporium of *Bacillus anthracis*. Microbiology 150:355–363

Richter D, Postic D, Sertour N, Livey I, Matuschka FR, Baranton G (2006) Delineation of *Borrelia burgdorferi* sensu lato species by Multilocus sequence analysis and confirmation of the delineation of *Borrelia spielmanii* sp. nov. Int J Syst Evol Microbiol 56:873–881

Sampath R, Hall TA, Massire C, Li F, Blyn LB, Eshoo MW, Hofstadler SA, Ecker DJ (2007) Rapid identification of emerging infectious agents using PCR and electrospray ionization mass spectrometry. Ann N Y Acad Sci 1102:109–120

Seo GM, Kim SJ, Chai YG (2004) Rapid profiling of the infection of *Bacillus anthracis* on human macrophages using SELDI-TOF mass spectroscopy. Biochem Biophys Res Commun 325:1236–1239

Stepanovic S, Hauschild T, Dakic I, Al-Doori Z, Svabic-Vlahovic M, Ranin L, Morrison D (2006) Evaluation of phenotypic and molecular methods for detection of oxacillin resistance in members of the *Staphylococcus sciuri* group. J Clin Microbiol 44:934–937

Vandamme B, Pot B, Gillis M, de Vos P, Kersters K, Swings J (1996) Polyphasic taxonomy, a consensus approach to bacterial systematics. Microbiol Rev 60:407–438

Versalovic J, Schneider M, de Bruijn FJ, Lupski JR (1994) Genomic fingerprinting of bacteria using repetitive sequence based PCR (rep-PCR). Methods Cell Mol Biol 5:25–40

Vos P, Hogers R, Bleeker M, Reijans M, van de Lee T, Hornes M, Freijters A, Pot J, Peleman J, Kuiper M, Zabeau M (1995) AFLP: a new concept for DNA fingerprinting. Nucleic Acids Res 21:4407–4414

Walker RD (2007) Antimicrobial susceptibility testing and interpretation of results. In: Giguere S, Prescott JF, Baggot JD, Walker RD, Dowling PM (eds) Antimicrobial therapy in veterinary medicine. Blackwell, Ames, IA

Wilson KH, Wilson WJ, Radosevich JL, DeSantis TZ, Viswanathan VS, Kuczmarski TA, Andersen GL (2002) High-density microarray of small-subunit ribosomal DNA probes. Appl Environ Microbiol 68:2535–2541

World Organization for Animal Health (OIE) (2010) Chapter 6.9. Responsible and prudent use of antimicrobial agents in veterinary medicine. In: OIE terrestrial animal health code, vol 1. OIE, Paris, France

Zeigler DR (2003) Gene sequences useful for predicting relatedness of whole genomes in bacteria. Int J Syst Evol Microbiol 53:1893–1919

Zelazny AM, Ferraro MJ, Glennen A, Hindler JF, Mann LM, Munro S, Murray PR, Reller LB, Tenover FC, Jorgensen JH (2005) Selection of strains for quality assessment of the disk induction method for detection of inducible clindamycin resistance in staphylococci: a CLSI collaborative study. J Clin Microbiol 43:2613–2615

Chapter 14
Continuous Elution Electrophoresis: A Unique Tool for Microbial Protein Analysis

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Abstract Microbial proteins are diverse and fascinating matter to study. Majority of time it happens that a protein cannot be studied at fine details as its properties are masked by others in vicinity. For the same, purification to homogeneity is required, and there are enormous number of techniques for protein purification currently followed in various laboratories. We will discuss one of them here. This chapter focuses on purification of protein sample from fungal sources to homogeneity by continuous elution electrophoresis (CEE) using Miniprep Cell. This is an extremely reproducible technique for getting single protein fractions during purification steps and works beyond doubt for separating two different proteins which resemble each other in all respect except a difference of few Daltons. In the present review, we will highlight the importance of continuous elution electrophoresis in separating biologically active proteins.

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14.1 Introduction

In spite of varied research, large-scale analysis of protein complexes still remains one of the major challenges of proteomics (Huang et al. 2009). Fractionation of protein complexes that are compatible with downstream processing is required. Micropreparative approach has the capacity to separate milligram quantities of protein complexes, which can assist the study of proteins of lower abundances. Polyacrylamide gel electrophoresis separates molecules in complex mixtures, and during electrophoresis there is an intricate interaction of samples, gel matrix buffers, and electric current resulting in separate bands of individual molecules. Empirically the pore size providing optimum resolution for proteins is that which results in the relative mobility (R*f*) value between 0.55 and 0.6. Polyacrylamide gel electrophoresis when done in the presence of sodium dodecyl sulfate ensures dissociation of proteins into their individual polypeptide subunits and that minimizes aggregation and accounts for proper separation of protein subunits (Laemmli 1970). These molecules separated by SDS-PAGE are biologically inactive, and the process accounted for separating proteins along with maintaining its activity is native PAGE, where the protein mobility is determined by its size and charge. This technique makes use of varied buffer system and is highly dependent on p*I* of protein of interest and pH of electrophoresis buffer (Ornstein and Davis 1964). Protein separation at preparative level can be performed either by scale-up of the analytical SDS-PAGE procedure or by continuous elution electrophoresis through Mini Prep Cell (Fig. 14.1) from Bio-Rad (Bio-Rad Laboratories, Hercules, CA, USA). Mini Prep Cell is a beneficial tool as compared to polyacrylamide gel electrophoresis as scale-up of the analytical SDS-PAGE does not have high resolving power and results in insufficiently purified material, and further with the Miniprep Cell, proteins differing by 2 % in molecular weight can be separated within 6 h (Lee and Harrington 2002).

Continuous elution electrophoresis (CEE) has been used as a preparative tool for blot overlay analysis (Mulvey and Ohlendieck 2003). Murray described a preparative method for starch gel electrophoresis in which electrophoretically separated components of the mixture can be eluted directly and continuously from a starch gel (Murray 1962). Preparative electrophoresis devices fractionate and purify nanogram to gram quantities of proteins and nucleic acids by electrophoresis and CEE (Bio-Rad Laboratories).

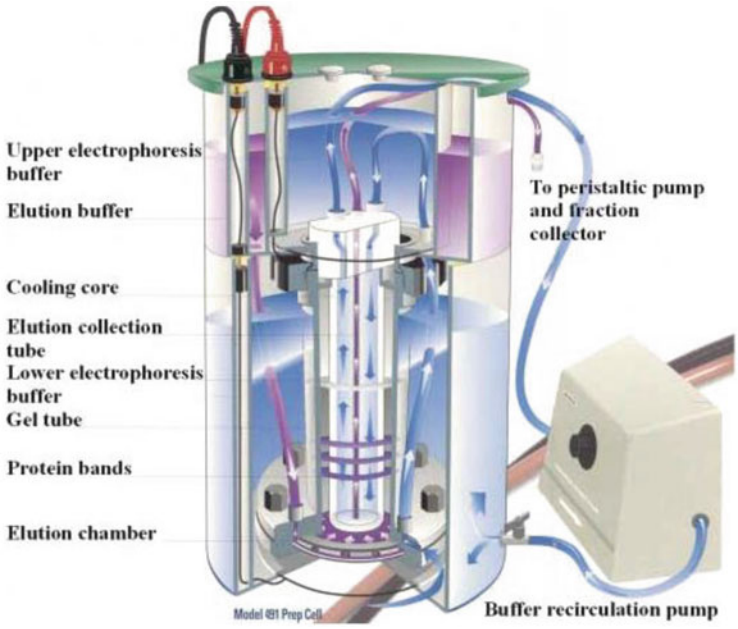

Fig. 14.1 Electrophoresis chamber *Model 491 Prep Cell* from Bio-Rad for isolating protein molecules in complex biological matrices (*Bio-Rad Laboratories, Inc.*)

14.2 Blue Native Continuous Elution Electrophoresis as Primary Micropreparative Fractionation Technique

Various studies have reported the use of CEE as primary micropreparative fractionation technique. Blue native continuous elution electrophoresis is a combination of blue native PAGE and CEE and has been used by Huang and his group for generation of protein complexes of about 800 kDa (Huang et al. 2009).

Continuous elution polyacrylamide gel electrophoresis procedure was used to purify a recombinant enzyme from *Dictyostelium* expressed as inclusion body in expression host BL-21 CodonPlus (DE3)-RIL. The enzyme was obtained at a yield of 5 mg/l of culture broth, with a specific activity of 0.7 nmol/min/mg protein (Ubeidat and Rutherford 2003). In a previous study Ubeidat and Rutherford developed expression system and single-step purification method for developmentally regulated protein from *Dictyostelium discoideum* (Ubeidat and Rutherford 2002).

They purified the recombinant protein to homogeneity using CEE and could obtain 10 mg of protein per liter growth medium.

14.3 CEE as Secondary Preparative Tool

CEE clubbed with other chromatographic and electrophoretic tools can be widely used for purification of complex proteins to homogeneity. A 23 kDa endo-β-xylanase from *Thermomyces lanuginosus* NCIM 1374/DSM 28966 was purified to homogeneity using gel filtration and ion-exchange chromatography followed by continuous elution electrophoresis (Figs. 14.2 and 14.3) (Shrivastava et al. 2011).

Fusion proteins of human immunodeficiency virus type I (HIV-1), gp-41, obtained from cells overexpressing virus envelope protein and HIV isolates were purified using preparative scale purification technique where proteins were extracted by sodium dodecyl sulfate polyacrylamide gel electrophoresis and then subjected to separation by CEE using zwitterionic detergent in elution buffer (Viard et al. 2002). This approach proved very beneficial for isolation of integral membrane proteins that undergo spontaneous folding and aggregation.

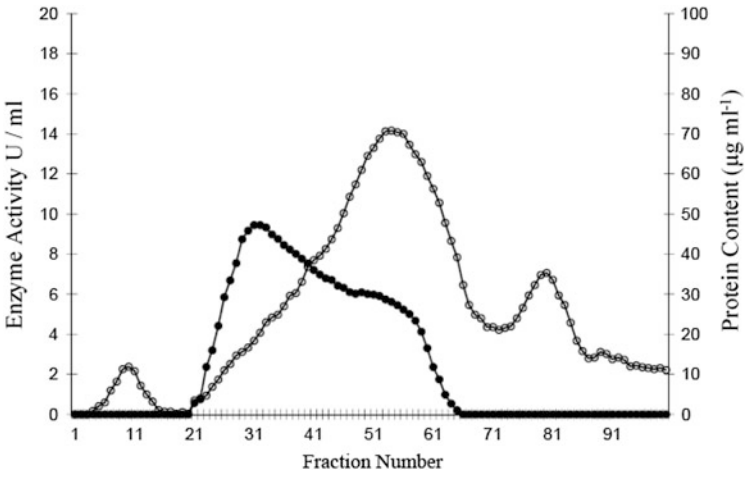

Fig. 14.2 Elution profile of xylanase from *Thermomyces lanuginosus* on continuous elution electrophoresis native polyacrylamide (15 %) gel. Xylanase activity (*filled circle*) and total protein absorbance at 280 nm. (*Unfilled circle*) Fraction volume of 1 ml (Shrivastava et al. 2011)

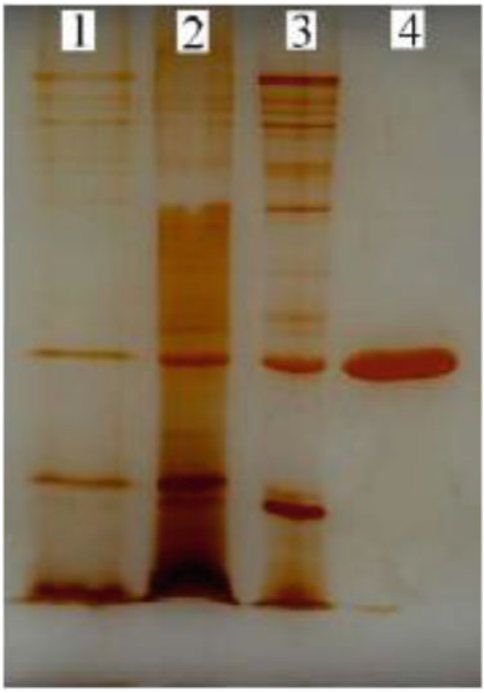

Fig. 14.3 Silver-stained SDS-PAGE (18 %) of xylanase purification steps. *Lane 1*, crude extract; *lane 2*, ultrafiltration retentate; *lane 3*, anion-exchange chromatography xylanase positive elute; *lane 4*, purified xylanase after continuous elution electrophoresis (Shrivastava et al. 2011)

14.4 CEE and Blot Overlay Techniques

Blot overlay technique in general was used to visualize protein-protein interaction on membrane and had a major drawback that it could not work efficiently in limited quantities of protein. This technique when clubbed with CEE could be utilized for rapid identification of protein-protein interaction. Mulvey and Ohlendieck separated microsomal muscle proteins ranging from 60 to 200 kDa electrophoretically and identified them based on immunoblotting of marker proteins. Thus it was seen that conjugation of CEE and blotting technique resulted in rapid identification of protein-protein interaction and could lead to future high-throughput analysis of subproteomes (Mulvey and Ohlendieck 2003).

14.5 Stepwise Elution Chromatography

Yamamoto and his team designed a method of stepwise elution chromatography which was effective for protein purification as well as its concentration. Stepwise elution chromatography could concentrate α-amylase by 30-fold and purify it by a factor of 23-fold (Yamamoto et al. 1992). A similar study on optimization of eluted salt concentration was applied to the actual protein separation system for recovery

of monoclonal antibodies, and a successful separation system was designed using optimized condition (Ishihara et al. 2006). A similar study on stepwise elution chromatography was done based on linear gradient elution experiment. This experimental setup could separate β-galactosidase using medium-performance (an)ion-exchange chromatography (Yamamoto et al. 1992). This group had also done extensive study on resolution of protein in linear gradient elution ion-exchange and hydrophobic interaction chromatography (Yamamoto et al. 1987). Yamamoto and Kita also gave a rational design calculation method for stepwise elution chromatography of proteins by which the efficiency of process was enhanced, and separation time was highly reduced (Yamamoto and Kita 2006).

14.6 Conclusions

Continuous elution electrophoresis individually as well as when clubbed with other techniques like blotting, blot overlay, and chromatography, etc. has proved to be efficient tool for protein and nucleic purification at preparative scale. Protein concentration could further be enhanced using stepwise elution chromatography (Yamamoto et al. 1992). On comparison of various studies, it was found that the amount of protein recovered after purification was higher when CEE was used as a single tool for the process, rather than when clubbed with other techniques (Shrivastava et al. 2011; Ubeidat and Rutherford 2002, 2003). Micropreparative fractions have been used for various analyses including processes like the study of *Saccharomyces cerevisiae* cellular lysate (Huang et al. 2009). From various studies reporting continuous elution electrophoresis, it could be very well driven that this technique has proved to be of potential use for protein purification with high yield and fast recovery, and its further utilization can give us wide understanding of many unexplored microbial proteins.

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References

Huang KY, Filarsky M, Padula MP, Raftrey MJ, Herbert BR, Wilkins MR (2009) Micropreparative fractionation of the complexome by blue native continuous elution electrophoresis. Proteomics 9:2494–2502

Ishihara T, Kadoya T, Endo N, Yamamoto S (2006) Optimization of elution salt concentration in stepwise elution of protein chromatography using linear gradient elution data: reducing residual protein A by cation-exchange chromatography in monoclonal antibody purification. J Chromatogr A 1114:97–101

Laemmli UK (1970) Cleavage of structural proteins during the assembly of head of Bacteriophage T4. Nature 227:680–685

Lee KH, Harrington MJ (2002) Preparative gel electrophoresis. In: Hames BD (ed) Gel electrophoresis of proteins: a practical approach. Oxford University Press, New York, pp 97–98

Mulvey C, Ohlendieck K (2003) Use of continuous-elution gel electrophoresis as a preparative tool for blot overlay analysis. Anal Biochem 319:122–130

Murray K (1962) A continuous elution method of preparative starch gel electrophoresis. Anal Biochem 3:415–428

Ornstein L, Davis BJ (1964) Disc electrophoresis I- Background and theory. Ann N Y Acad Sci 121:321–349

Shrivastava S, Shukla P, Mukhopadhyay K (2011) Purification and preliminary characterization of a xylanase from *Thermomyces lanuginosus* strain SS-8. 3 Biotech 1:255–259. doi:10.1007/s13205-011-0032-6

Ubeidat M, Rutherford CL (2003) Purification and renaturation of *Dictyostelium* recombinant alkaline phosphatase by continuous elution electrophoresis. Protein Expr Purif 27:375–383

Ubeidat M, Rutherford CL (2002) Expression and one-step purification of developmentally regulated protein from *Dictyostelium discoideum*. Protein Expr Purif 25:472–480

Viard M, Blumenthal R, Raviv Y (2002) Improved separation of integral membrane proteins by continuous elution electrophoresis with simultaneous detergent exchange: application to the purification of the fusion protein of the human immunodeficiency virus type 1. Electrophoresis 23:1659–1666

Yamamoto S, Kita A (2006) Rational design calculation method for stepwise elution chromatography of proteins. Food Bioprod Process 84:72–77

Yamamoto S, Nomura M, Sano Y (1987) Resolution of proteins in linear gradient elution ion-exchange and hydrophobic interaction chromatography. J Chromatogr A 409:101–110

Yamamoto S, Nomura M, Sano Y (1992) Stepwise elution chromatography as a method for both purification and concentration of proteins. Chem Eng Sci 47:185–188

Chapter 15
Detection and Characterization of Endobacteria in the Fungal Endophyte *Piriformospora indica*

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Abstract The nonpathogenic Alphaproteobacterium *Rhizobium radiobacter* (syn. *Agrobacterium tumefaciens*) (*Rr*F4) is a subculture of the endobacterium *R. radiobacter*, which is intricately associated with its host, the beneficial plant-colonizing fungal basidiomycete *Piriformospora indica*. *Rr*F4 is genetically very similar to the well-studied plant pathogenic *R. radiobacter* biovar I strain C58 (genomovar G8). Highly similar genetic content of *Rr*F4 and C58 denotes the high potential for *Rr*F4 to directly interact with plants. The failure to cure *P. indica* from its endobacterium still hampers a conclusive prediction of the bacterium's extended role in the interaction of the fungus with a broad spectrum of host plants. However, beneficial activities shown in cereals and the Brassicaceae *Arabidopsis thaliana* were hardly distinguishable when induced either by *Rr*F4 or *P. indica*. We discuss here the various strategies employed to detect, characterize, and eventually elucidate the endobacterium's role in the tripartite symbiosis with its fungal host and a plant.

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15.1 Biologically Characterized Interactions of Higher Fungi with Endobacteria

Although soilborne beneficial microbes are prevalent, their phylogenetic, spatial, and functional diversity is widely unresolved (Berg et al. 2014). Complex symbiotic interactions including bacteria, fungi, and their host plants are especially little understood, and their global prevalence is widely unknown (Lackner et al. 2009; Hoffman and Arnold 2010; Naumann et al. 2010). Endobacteria in higher fungi were previously detected in Glomeromycotan arbuscular mycorrhizal symbioses (Bonfante and Anca 2009; Naumann et al. 2010), in the ectomycorrhizal fungus *Laccaria bicolor* (Bertaux et al. 2003; Bertaux et al. 2005), in the rice pathogenic fungus *Rhizopus microsporus* (Partida-Martinez and Hertweck 2005), in hyphae of phylogenetically diverse foliar fungal endophytes (Hoffman and Arnold 2010), in the soil isolate *Mortierella alpina* (Kai et al. 2012), and in plant symbiotic Endogone *Mucoromycotina* fungi (Desirò et al. 2015).

We studied the tripartite Sebacinalean interaction, comprising fungi of the order *Sebacinales* (Basidiomycota), phylogenetically diverse endofungal bacteria, and a broad range of monocotyledonous and dicotyledonous host plants (for a review, see Qiang et al. 2012a). A first analysis of the distribution on a worldwide scale suggested that Sebacinalean symbioses are prevalent in all continents (Weiss et al. 2011; Reiss et al. 2014), potentially making them a vital part of global soil ecosystems. The root endophyte *Piriformospora indica* (syn. *Serendipita indica*) is a model fungus of the *Serendipitaceae* of the *Sebacinales* (Oberwinkler et al. 2014). Since its discovery in the Indian Thar dessert in 1996 (Varma et al. 1999), *P. indica* have been shown to promote biomass, yield, and health of a broad spectrum of plants (Varma et al. 2012; Ye et al. 2014; Peškan-Berghöfer et al. 2004; Waller et al. 2005; Deshmukh et al. 2006; Camehl et al. 2010; Qiang et al. 2012b). Genetic and biochemical assessments of the resistance mechanism that is induced by *P. indica* in *Arabidopsis thaliana* against a wide spectrum of leaf and root pathogens showed a need for an operable jasmonate- but not salicylate-dependent pathway and thus is consistent with an induced systemic resistance (ISR)-type response (Stein et al. 2008; Jacobs et al. 2011). Intriguingly, despite the fact that *P. indica* mediates resistance to root and leaf pathogens, several reports have shown that the fungus suppresses part of the roots' defense systems for a successful colonization (Jacobs et al. 2011). For instance, the fungus exploits the antagonism of the SA–JA cross talk to downregulate SA-linked defense by upregulating the jasmonate pathway. Concomitantly, colonization by the mutualistic fungus induces the antioxidant system which supports its own early biotrophic phase and by the

same time leads to a strong protection of root tissue against necrotrophic challenger (Baltruschat et al. 2008; Harrach et al. 2013). It is reasonable that a combination of such efficient immune modulating strategies eventually evolved the capacity to colonize an unprecedented broad range of host plants.

Members of the *Serendipitaceae* regularly undergo complex symbioses involving plants and endofungal bacteria of different genera (Sharma et al. 2008). So far detected endobacteria, associated with fungi of the genera *Piriformospora* and *Sebacina*, belong to two genera of Gram-negative (*Rhizobium* and *Acinetobacter*) and two genera of Gram-positive (*Paenibacillus* and *Rhodococcus*) bacteria. The best-studied example of a tripartite Sebacinalean symbiosis is the association of *P. indica* with the Alphaproteobacterium *Rhizobium radiobacter* (syn. *Agrobacterium radiobacter;* syn. *Agrobacterium tumefaciens*). Fluorescence in situ hybridization (FISH) using universal bacteria (EUB) and a *Rhizobium*-specific 16S rRNA targeting probe (Rhi1247) confirmed the stable endocellular association of small numbers of bacterial cells within *P. indica* chlamydospores and hyphae (Sharma et al. 2008). A recent assessment of the relative abundance of bacterial cells in *P. indica* during colonization of barley root tissues by quantitative (q)PCR targeting the internal transcribed spacer (ITS) between the 16S rRNA and 23S rRNA gene of the ribosomal RNA operon of *R. radiobacter* and the fungal *tef* gene (Glaeser et al. 2016) detected one bacterial cell per 450 *P. indica* nuclei of the multinucleated fungus. Importantly, the number of bacteria per fungal unit—as normalized by the fungal *tef* gene—varied greatly depending on the condition used to cultivate the fungus. Highest numbers of bacterial cells were detected in fungal mycelium freshly isolated from surface-sterilized roots. Consistent with this, the number of bacterial cells also significantly increased when liquid *P. indica* cultures were supplemented with root material (Guo, Glaeser unpublished).

15.2 Attempts to Cure *P. indica* from Endobacteria

Piriformospora indica is the only Sebacinales fungus axenically cultivable bacteria could have been isolated from. The endobacterium was isolated from powdered fungal mycelia of *P. indica* (DSM 11827, Sharma et al. 2008) and propagated in axenic cultures for a prolonged period. A subculture of the original isolate *R. radiobacter* strain PABac-DSM (Sharma et al. 2008) was used for detailed studies of the interaction of the isolated bacterium with various plants. This subculture was designated *R. radiobacter* F4 (*Rr*F4; Glaeser et al. 2016).

Importantly, while the bacterium could be isolated from *P. indica*, various attempts to cure the fungus of its resident endobacterial cells have failed. Experiences with other endofungal bacteria suggested two possible strategies for curing attempts: *i*. Antibiotics were successfully used to kill endofungal bacteria in the

rice-pathogenic zygomycete *Rhizopus microsporus*, thereby obtaining a cured fungus (e.g., Partida-Martinez and Hertweck 2005). *ii*. The arbuscular mycorrhiza (AM) fungus *Gigaspora margarita* could not be cured by antibiotic treatment but repeated passages through single-spore inocula resulting in a bacteria-free fungal line (Lumini et al. 2007). In both examples, fungi survived curing, though *G. margarita* showed a reduced fitness. In order to generate a bacterium-free *P. indica*, we extended earlier attempts (Sharma et al. 2008) by the following strategies: Axenic *P. indica* cultures were treated with a mixture of the antibiotics spectinomycin and ciprofloxacin that are known to be very efficient in killing *Rr*F4 in liquid bacterial cultures. Protoplasts were isolated from this mycelium and cultured under the same antibiotics to avoid that the fungal cell wall may hinder the entrance of antibiotics. After three rounds of clonal selection of fungal protoplasts, bacteria could no longer be detected in regenerating mycelia by qPCR. Germination of chlamydospores derived from these fungal clones was delayed, and single colonies were approximately 50 % smaller in size and produced less spores compared to colonies grown without antibiotics. However, upon colonization of plant roots always resulted in a recovery of bacteria, showing that they were not completely removed by the antibiotic treatments. The fact that bacteria could not be killed inside the hyphae by antibiotics that were very efficient on isolated bacterial cultures (Sharma et al. 2008) raised the hypothesis that endofungal *R. radiobacter* under certain environmental conditions enters an inactive state where it becomes non-cultivable and also might be insensitive to antibiotics. The bacteria may have either shifted to a viable but non-culturable (VBNC) state as suggested by Oliver (2005) or a dormant state with low metabolic activity and extended period without cell division (Kaprelyants et al. 1993). Both physiological states would cause resistance against antibiotics. Differences in cell morphology of the free-living bacterium (large rod-shaped cells) and the fungal endobacteria (small coccoid rods) support this hypothesis as cell size reduction is one of the main criteria of those physiological stages. Mycelium that was treated with antibiotics and thus showed reduced spore production was termed "partially cured" *P. indica* (pcPIRI). Other strategies to cure the fungus included maintaining and multiplying the axenic fungus under antibiotics, clonal propagation from single fungal chlamydospores, and selective isolation of fungal mycelia from youngest hyphae in radial mycelial clones. In all these cases, bacteria cell numbers initially declined down to the qPCR detection level, but eventually recovered after a colonization of roots in the absence of antibiotics. Importantly and consistent with an intricate association of *P. indica* and *R. radiobacter*, no other bacterial species have been isolated from worldwide distributed laboratory cultures of *P. indica*.

15.3 Analysis of the *Rr*F4 Genome

The genome of *Rr*F4 was sequenced and compared to related pathogenic *Agrobacterium* strains (Glaeser et al. 2016). Genomic DNA was collected and shotgun libraries were prepared for sequencing. Emulsion PCR (emPCR), emulsion breaking of DNA-enriched beads, and sequencing of the shotgun libraries were performed on a second-generation pyrosequencer (454 GS FLX Titanium) using Titanium reagents and Titanium. Quality filtering of the pyrosequencing reads was performed using the automatic standard signal processing pipeline of the Roche GS Run Processor to remove failed and low-quality reads from raw data and to remove adaptor sequences. The initial assembly of the data from 454 pyrosequencing was performed using the Roche GS FLX Newbler software 2.0.01 with a minimum overlap length of 40 bp and a minimum overlap identity of 90 % followed by a comparative alignment to the genome of *R. radiobacter* (syn. *A. tumefaciens, A. radiobacter*) C58 which resulted in four distinct contigs. The data were uploaded into GenDB (Meyer et al. 2003) and subjected to an automatic annotation. BLAST ring images were generated using BRIG (Alikhan et al. 2011). Genome comparisons were performed in EDGAR (Blom et al. 2009; Blom et al. 2016). This Whole Genome Shotgun project has been deposited at DDBJ/EMBL/GenBank under the accession JZLL00000000 (Glaeser et al. 2016). Using this technical strategy, we found a very high degree of similarity of *Rr*F4 to the genome of the plant pathogenic C58 (Goodner et al. 2001). Like C58, *Rr*F4's genome is organized in a circular (2.8 Mb) and a linear chromosome (2.06 Mb), a tumor-inducing plasmid pTiF4 (0.21 Mb), and an accessory plasmid pAtF4 (0.54 Mb). The two strains share 95 % of the genes from the circular and 98 % of the genes from the linear chromosomes. The average amino acid identity (AAI) of shared genes is 99.8 % (Glaeser et al. 2016), indicating the high genetic relationship of the two bacteria. At a taxonomic point of view, the two strains clearly belong to the same species. Based on the phylogenetic assignment of C58 and *Rr*F4, they are members of the *Agrobacterium* genomovar G8 which was indicated by extended comparative analysis of the two genomes (Glaeser et al. 2016).

Interestingly, the circular and linear chromosomes had 100 and 80 singleton Coding Sequences (CDs), respectively, not present in C58. Most of these CDs are of unknown function and future analysis must show whether these genes represent candidates for supporting the endofungal growth of *Rr*F4 and/or fitness of its fungal partner *P. indica*. The genetic alterations among *Rr*F4 and C58 in both the pTi and pAt plasmids such as the loss of the T-DNA can explain the loss of *Rr*F4's pathogenicity (Glaeser et al. 2016). In contrast to the non-cultivable endobacterium *Cand.* Glomeribacter gigasporarum, which was isolated from the AM fungus *Gigaspora margarita*, the genome of *Rr*F4 is not reduced. This is reminiscent of the cultivable endobacterium *Burkholderia rhizoxinica* that also possess a merely

slightly reduced genome (Lackner et al. 2011) as compared to free-living *Burkholderia* species. Thus, the data are consistent with the hypothesis that *R radiobacter* forms a facultative symbiosis with *P. indica*, where the bacterium still is able to live independently of its host (Glaeser et al. 2016).

While *Rr*F4 and C58 showed a high degree of similarity based on the circular and linear chromosomes, the plasmids were more diverse (Glaeser et al. 2016). These differences partly stem from gene translocations in *Rr*F4 from the pTi to pAt plasmid, partly from unique sequences in both strains. The *acc* operon, responsible for agrocinopine A+B uptake and catabolism, the *arc* operon for agrocinopine regulation of conjugation, a truncated version of the *virH1* gene, and the *tra* operon described by Piper et al. (1999) were translocated in *Rr*F4 from plasmid pTi to pAt. The latter one also includes the quorum sensing regulator gene *traR* but not the AHL synthase *traI*, which remains upstream of the *trb* operon on pTi in both strains. Furthermore, *Rr*F4 harbors an additional set of *tra* genes on the pTiF4 plasmid which shows highest similarity to the conjugation genes on the RP4 plasmid of *Escherichia coli*. Most interestingly, in contrast to C58, *Rr*F4 lacks the complete transfer DNA (T-DNA) region and some adjacent genes belonging to the nopaline catabolic (*noc*) region. It is hard to speculate which role the additional *E. coli*-like *tra* genes might play for the nonpathogenic *Rr*F4. While the "*Rhizobium*-like" *tra* genes are usually involved in the transfer of the T-region to the infected plant cells and are regulated by a *luxI*/*luxR*-type quorum sensing system (Fuqua and Winans 1994), the *E. coli tra* genes are required for bacterial conjugation (Frost et al. 1994).

Although it shares nearly identical circular and linear chromosomes with the pathogenic *A. tumefaciens* C58, *Rr*F4 is nonpathogenic. As shown previously, and consistent with our finding, curing C58 from its pTi plasmid results in a nonpathogenic strain with albeit weak plant growth-stimulating activity (Walker et al. 2013). Nevertheless, at present we do not know all the factors needed by *Rr*F4 to associate with its fungal host *P. indica*. Lackner et al. (2011) denoted the intrahyphal *Burkholderia rhizoxinica* as a bacterium with a "genome in transition" because it showed, at least to some extent, a reduced genome size compared to free-living *Burkholderia* species. While *Rr*F4 did not show a reduced genome size, changes in the structure and gene content of both the pTi and pAt plasmids may hint to an adaptation to a specific ecological niche. Especially the shift of several genes from the pTi to the pAt plasmid could be a hint for an evolutionary adaptation from a pathogenic to a symbiotic lifestyle. The gene content alone, however, will not be sufficient to explain the endofungal lifestyle of *Rr*F4; thus, further studies must elucidate the molecular communication in the tripartite association between bacterium, fungus, and plant.

15.4 *Rr*F4 Colonizes Roots and Proliferates Independent of its Fungal Host

Free-living *Rr*F4 colonizes plant roots and multiples independently of its natural fungal host *P. indica*. Since axenically grown plants are most suitable for quantification and microscopy of bacteria in association with roots, plant seedlings were grown on half-strength Murashige & Skoog (MS) medium. To produce an optimal inoculum, *Rr*F4 was grown overnight in modified LB broth (1 % casamino hydrolysate, 0.5 % yeast extract, and 0.5 % NaCl, pH 7.0, supplemented with 100 µg mL^{-1} gentamycin) at 28°C and 150 rpm. Bacterial cells were collected by centrifugation (3202 g, 10 min) and washed and resuspended in 10 mM $MgSO_4$ $7H_2O$ buffer. Roots of three-day-old barley/wheat seedlings or seven-day-old Arabidopsis seedlings, respectively, were dip inoculated for 30 min in *Rr*F4 suspensions (OD_{600} = 1.0 to 1.4). Control seedlings were dipped into 10 mM $MgSO_4$ $7H_2O$. At various time points after dip inoculation, root material was collected from the medium and *Rr*F4 was quantified by qPCR targeting the ITS of the ribosomal RNA operon. Before DNA extraction, roots were sequentially washed in 70 % ethanol and distilled water and sonicated to remove excess of bacteria from the surface. Upon this treatment, the amount of *Rr*F4 cells that remained attached to the Arabidopsis roots was below the qPCR detection limit. During two weeks, *Rr*F4 cells proliferated in the apical 4 cm of the roots as indicated by a significant increase in *Rr*F4 ITS targets relative to plant *ubiquitin*. At 14 dpi, bacterial cell numbers reached 2.9 [±0.5] × 10^9 cells per g Arabidopsis root FW (Glaeser et al. 2016). Interestingly, *Rr*F4 propagated even better in roots of barley and wheat seedlings: higher ratio values for ITS targets vs. root gene targets suggested a more intense bacterial colonization of the monocotyledonous plants. Moreover, high amounts of *Rr*F4 cells remained attached to the root surface even directly after dip inoculation [0 dpi; barley: 2.1 (±0.4) × 10^8; wheat: 3.3 (±2.0) × 10^8 cells per g FW of roots, which let us speculate that *Rr*F4 bacteria interact more efficiently with the surface of graminaceous plants than with Arabidopsis. At 14 dpi, bacterial cell numbers reached 8.1 [±1.4] × 10^9 (barley) and 6.0 [±2.5] × 10^9 (wheat) per gram root FW, suggesting that *Rr*F4 interacts and multiplies in barley and wheat with high efficiency (Glaeser et al. 2016).

15.5 *Rr*F4 Multiplies at the Root Surface

Colonization of barley roots was also assessed microscopically with GFP-tagged bacteria (Fig. 1; Glaeser et al. 2016). At five days after dip inoculation, *Rr*F4 cells were seen in the maturation zone I of primary roots in an area covering approximately one cm in length (Fig. 15.1a). At 14 dpi, bacteria had spread into the maturation zone II, while elongation and meristematic zones as well as the root cap remained virtually free of bacteria (Fig. 15.1b). This colonization pattern

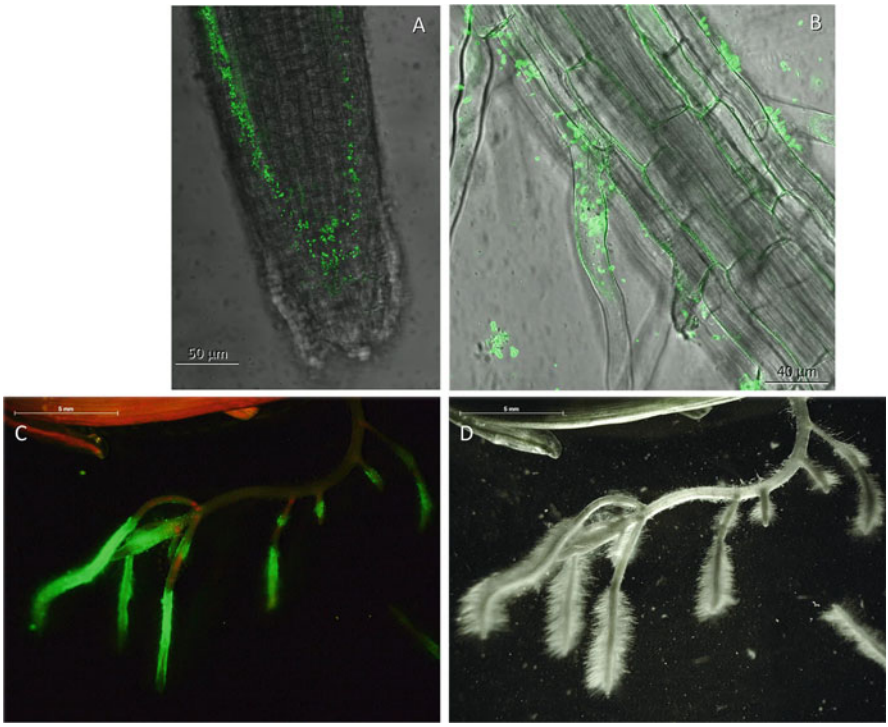

Fig. 15.1 Localization of GFP-expressing *Rr*F4 in Arabidopsis roots. *Rr*F4 on a primary root at 7 (**a**) and 14 dpi (**b**) and on lateral roots at 30 dpi (**c**, **d**). GFP-expressing *Rr*F4 are visualized by epifluorescence microscopy

resembled the root colonization pattern of *P. indica* (Jacobs et al. 2011). Microscopic analysis also revealed a distinct pattern in the root hair zone, suggesting specific sites of higher *Rr*F4 proliferation at the root surface. At later time points, root hair zones of lateral roots also were colonized with the same pattern as in primary roots (Fig. 15.1c,d). Bacterial conglomerates were particularly present at lateral root protrusions that probably serve as entry sites into the central cyclinder (see Sect. 15.6). Scanning electron microscopy detected conglomerates of *Rr*F4 cells on root surface (Fig. 15.2). Bacterial cells formed biofilms as evidenced by extracellular fiber-like structures. Inoculation of wheat and Arabidopsis roots with *Rr*F4 virtually showed the same colonization pattern and accordingly also resembled the pattern observed with *P. indica*.

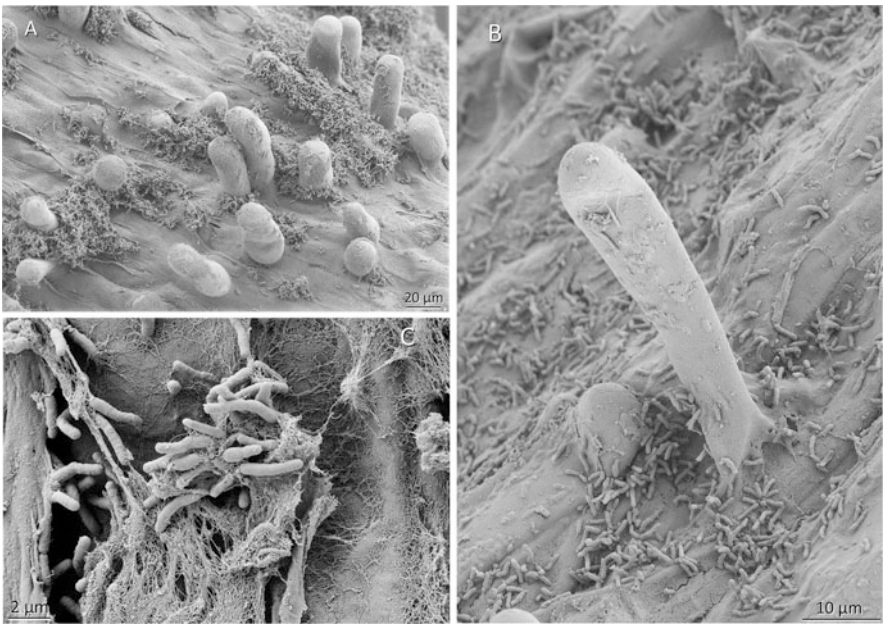

Fig. 15.2 Colonization of barley primary roots by *Rr*F4 analyzed by scanning electron microscopy. (**a**, **b**) *Rr*F4 at the root hair zone. (**c**) *Rr*F4 cell aggregates at the root tip. Bacterial cells are cross-linked by fiber-like structures

15.6 *Rr*F4 Cells were Detected in the Intercellular Space of the Root Cortex and in the Central Cylinder

Cross sections of barley roots were analyzed for bacteria colonizing the inner root. About one week after dip inoculation, *Rr*F4 cells were visible in the root cortex. Bacteria were often detected at cell junctions of rhizodermal and cortical cells. Light microscopy and TEM of ultrathin root cross sections showed a dense colonization of the intercellular spaces in the cortex tissue up to the endodermis. Root hair cells and cortex cells were not colonized, which implies an extracellular colonization pattern (Glaeser et al. 2016).

At later time points, *Rr*F4 cells were also detected at cell junctions of endodermal cells and in the central cylinder. Inside the central cylinder, bacteria were also seen in the intercellular spaces while intracellular colonization was not unambiguously detectable. This finding is crucial insofar as colonization of the inner part of the root beyond the endodermis has not been observed with *Rr*F4's fungal host *P. indica* (Deshmukh et al. 2006; Jacobs et al. 2011). However, *Rr*F4 cells seem not to spread systemically into the upper plant tissue, as bacteria could not be detected in stems and leaves, neither by cultivation nor by qPCR detection.

15.7 Cell Death Associated Colonization Strategies

In Arabidopsis roots, *P. indica* consecutively exhibits a biotrophic and a cell death-associated colonization strategy (Deshmukh et al. 2006). In the latter stage, the fungus invades root cells by inducing an endoplasmic reticulum (ER) stress-triggered caspase-dependent cell death (Qiang et al. 2012b). In contrast, *Rr*F4 cells were detected in the extracellular space, especially in the cortically tissue, without evidence for an induced cell death stage. However, GUS-tagged *Rr*F4 bacteria also colonized dead rhizodermis cells as evidenced by light microscopy and TEM analysis (Glaeser et al. 2016). We speculate that single bacteria may have invaded such dead plant cells using cell-derived compounds as carbon sources for further propagation. Dead plant cells are a specific ecological niche where bacteria can get nutrients and can propagate in a protected environment. Whether the heavily colonized plant cells may have a further function for the interaction of *Rr*F4 with the plant is not known and hard to speculate.

15.8 *Rr*F4 Has an Operable Quorum Sensing System

In plant bioassays, *Rr*F4 showed beneficial activities, such as growth-promoting activity and induced resistance, widely comparable to those exerted by its fungal host *P. indica* on a broad range of plants (Glaeser et al. 2016). Further genetic analysis, including Arabidopsis mutants defective for various defense pathways, confirmed that *Rr*F4, like *P. indica*, mediates systemic resistance via the jasmonate-dependent ISR pathway (Glaeser et al. 2016).

In order to find bacterial metabolites that could mediate those beneficial activities, we conducted a chemical analysis of *Rr*F4-produced *N*-acyl-homoserine lactones (AHLs). Apart from its well-known function in bacterial communication, AHLs with acyl chains of C12 and C14 have strong resistance-inducing activity in mono- and dicotyledonous plants against a broad spectrum of microbial pathogens such as fungi and bacteria, while shorter chained AHLs possess morphogenic activity (Schikora et al. 2011; Schenk et al. 2014). Interestingly, our analysis revealed that *Rr*F4 produces AHLs preferably with acyl chains of C8, C10, and C12 as well as hydroxyl- or oxo-substitutions at the C3 position in liquid culture (Li 2011). To find genetic evidence for the requirement of AHL to colonize plant roots, the Hartmann lab produced an *Rr*F4 variant (*Rr*F4NM13) that overexpresses an AHL cleaving lactonase. Significantly, this AHL-depleted mutant was strongly compromised for root colonization. Consistent with this, the *Rr*F4-induced systemic resistance to the bacterial pathogen *Xanthomonas translucens* pv. *translucens* (*Xtt*) is reduced in *Rr*F4NM13-treated wheat compared to plants treated with *Rr*F4, and growth-promoting activity exerted by *Rr*F4 in Arabidopsis was greatly abolished with *Rr*F4NM13 (not published).

15.9 Analysis of the Complex Sebacinalean Transcriptome

In collaboration with the CeBiTec Bielefeld, Germany, we have performed RNA sequencing-based transcriptome analysis in the tripartite symbiosis using TruSeq© stranded mRNA library preparation kits (Illumina) with modifications and Illumina MiSeq and HiSeq 1500 systems. Axenically growing barley seedlings were *i*. non-inoculated (control), *ii*. dip inoculated with *P. indica* (containing the endobacterium [tripartite symbiosis]), or *iii*. dip inoculated with *Rr*F4. Eukaryotic (barley, *P. indica*) and prokaryotic (endobacteria, *Rr*F4) cDNA libraries were prepared using a specifically adapted library preparation pipeline to get the highest yield of transcripts of all symbiosis partners. Changes in the barley transcriptome (control *vs*. *P. indica*- or *Rr*F4-inoculated roots) being currently investigated to compare the transcriptional response of barley in the tripartite symbiosis and in the direct interaction with *Rr*F4 compared to non-inoculated control plants. The transcriptome of *Rr*F4 (free form isolated from *P. indica*) and the endobacterium *R. radiobacter* will further be determined to understand the biological activity of *R. radiobacter* in the direct interaction with barley and in the tripartite symbiosis. First deep sequencing of eukaryotic and prokaryotic cDNA libaries were successful for the detection of eukaryotic mRNA transcripts of barley and *P. indica*. Prokaryotic mRNA transcripts could be efficiently detected for *Rr*F4-colonizing barley, but the number of transcripts detected for the endobacterium *R. radiobacter* in the tripartite symbiosis was very low because of the high abundance of mitochondrial transcripts of *P. indica*. The sequencing success was possible by a stringent biochemical enrichment of the respective RNA types by the CeBiTec lab. However, the data also show that a deeper sequencing of the bacterial cDNA libraries is required as this portion was low, though clearly present in the sample from the tripartite interaction. That CeBiTec's RNAseq data are of extraordinary quality also shows that we could reproduce (and extend) the microarray analysis of Zuccaro et al. (2011), e.g., by showing that candidate genes of the ISR pathway are induced).

15.10 Summary and Outlook

Recent progress in understanding the potential functions of the endobacterium *R. radiobacter* in the tripartite symbiosis has come from investigations of the direct interaction of the isolated endobacterium with different host plants. Future work must focus on the understanding of the role of the endobacterium in the natural habitat, the tripartite symbiosis. This venture is challenging because of the low numbers of bacterial cells in lab cultures of *P. indica*. Nevertheless, new sequencing technologies along with microscopically imaging approaches suggest a critical role of *R. radiobacter* for supporting fitness and vegetative propagation of its host fungus. This is a first step in the elucidation of this fascinating model of the tripartite Sebacinalean symbiosis where an endophytic fungus harbors an

endobacterium that seems to be “awake” during fungal root colonization and may contribute to many of the observed beneficial effects produced on plants.

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References

Alikhan NF, Petty NK, Ben Zakour NL, Beatson SA (2011) BLAST Ring Image Generator (BRIG): simple prokaryote genome comparisons. BMC genomics 12:402. doi:10.1186/1471-2164-12-402

Baltruschat B, Fodor J, Harrach B, Niemczyk E, Schwarzinger I, Barna B, Gullner G, Zuccaro A, Schäfer P, Kogel KH, Skoczowski A (2008) Salt tolerance induced by the root endophyte *Piriformospora indica* is associated with a strong increase in antioxidants. New Phytologist 180:501–510

Berg G, Grube M, Schloter M, Smalla K (2014) Unraveling the plant microbiome: looking back and future perspectives. Front Microbiol 5:148. doi:10.3389/fmicb.2014.00148

Bertaux J, Schmid M, Hutzler P, Hartmann A, Garbaye J, Frey-Klett P (2005) Occurrence and distribution of endobacteria in the plant-associated mycelium of the ectomycorrhizal fungus *Laccaria bicolor* S238N. Environ Microbiol 7:1786–1795

Bertaux J, Schmid M, Prévost-Bourre NC, Churin JL, Hartmann A, Garbaye J et al (2003) *In situ* identification of intracellular bacteria related to *Paenibacillus* spp. in the mycelium of the ectomycorrhizal fungus *Laccaria bicolor* S238N. App Environ Microbiol 69:4243–4248

Blom J, Albaum SP, Doppmeier D, Pühler A, Vorhölter FJ, Jewski MZ et al (2009) EDGAR: A software framework for the comparative analysis of prokaryotic genomes. BMC Bioinformatics 10:154. doi:10.1186/1471-2105-10-154

Blom J, Kreis J, Spänig S, Juhre T, Bertelli C, Ernst C, Goesmann A (2016) EDGAR 2.0: an enhanced software platform for comparative gene content analyses. Nucleic acids research 44:22–28

Bonfante P, Anca IA (2009) Plants, mycorrhizal fungi, and bacteria: a network of interactions. Annu Rev Microbiol 63:363–383

Camehl I, Sherameti I, Venus Y, Bethke G, Varma A, Lee J et al (2010) Ethylene signalling and ethylene-targeted transcription factors are required to balance beneficial and nonbeneficial traits in the symbiosis between the endophytic fungus *Piriformospora indica* and *Arabidopsis thaliana*. New Phytologist 185:1062–1073

Deshmukh SD, Hückelhoven R, Schäfer P, Imani J, Sharma M, Weiss M et al (2006) The root endophytic fungus *Piriformospora indica* requires host cell death for proliferation during mutualistic symbiosis with barley. Proc Natl Acad Sci USA 103:18450–18457

Desirò A, Faccio A, Kaech A, Bidartondo MI, Bonfante P (2015) Endogone, one of the oldest plant-associated fungi, host unique *Mollicutes*-related endobacteria. New Phytol 205:1464–1472

Frost LS, Ippen-Ihler K, Skurray RA (1994) Analysis of the sequence and gene products of the transfer region of the F sex factor. Microbiol Rev 58:162–210

Fuqua WC, Winans SC (1994) A LuxR-LuxI type regulatory system activates Agrobacterium Ti plasmid conjugal transfer in the presence of a plant tumor metabolite. J Bacteriol 176:2796–2806

Glaeser SP, Imani J, Alabid I, Guo H, Kämpfer P, Hardt M, Blom J, Goesmann A, Rothballer M, Hartmann A, Kogel KH (2016) Non-pathogenic Rhizobium radiobacter F4 deploys plant beneficial activity independent of its host *Piriformospora indica*. ISME J 10:871–884

Goodner B, Hinkle G, Gattung S, Miller N, Blanchard M, Qurollo B et al (2001) Genome sequence of the plant pathogen and biotechnology agent *Agrobacterium tumefaciens* C58. Science 294:2323–2328

Harrach BD, Baltruschat H, Barna B, Fodor J, Kogel KH (2013) The mutualistic fungus Piriformospora indica protects barley roots from a loss of antioxidant capacity caused by the necrotrophic pathogen *Fusarium culmorum*. MPMI 26:599–604

Hoffman MT, Arnold AE (2010) Diverse bacteria inhabit living hyphae of phylogenetically diverse fungal endophytes. Appl Environ Microbiol 76:4063–4075

Jacobs S, Zechmann B, Molitor A, Trujillo M, Petutschnig E, Lipka V et al (2011) Broad-spectrum suppression of innate immunity is required for colonization of Arabidopsis roots by the fungus *Piriformospora indica*. Plant Physiol 156:726–740

Kai K, Furuyabu K, Tani A, Hayashi H (2012) Production of the quorum-sensing molecules N-acylhomoserine lactones by endobacteria associated with *Mortierella alpina* A-178. Chembiochem 13:1776–1784

Kaprelyants AS, Gottschal JC, Kell DB (1993) Dormancy in non-sporulating bacteria. FEMS Microbiol Rev 10:271–285

Lackner G, Möbius N, Scherlach K, Partida-Martinez LP, Winkler R, Schmitt I, Hertweck C (2009) Global distribution and evolution of a toxinogenic *Burkholderia-Rhizopus* symbiosis. Appl Environ Microbiol 75:2982–2986

Lackner G, Moebius N, Partida-Martinez LP, Boland S, Hertweck C (2011) Evolution of an endofungal lifestyle: Deductions from the *Burkholderia rhizoxinica* genome. BMC Genomics 12:210. doi:10.1186/1471-2164-12-210

Li D (2011) Phenotypic variation and molecular signaling in the interaction of the rhizosphere bacteria Acidovorax sp, N35 and *Rhizobium radiobacter* F4 with roots. Faculty of Biology, Ludwig Maximilian University München, Germany, Dissertation

Lumini E, Bianciotto V, Jargeat P, Novero M, Salvioli A, Faccio A, Bécard G, Bonfante P (2007) Presymbiotic growth and sporal morphology are affected in the arbuscular mycorrhizal fungus Gigaspora margarita cured of its endobacteria. Cell Microbiol 9:1716–1729

Meyer F, Goesmann A, McHardy AC, Bartels D, Bekel T, Clausen J et al (2003) GenDB--an open source genome annotation system for prokaryote genomes. Nucleic Acids Res 31:2187–2195

Naumann M, Schüssler A, Bonfante P (2010) The obligate endobacteria of arbuscular mycorrhizal fungi are ancient heritable components related to the *Mollicutes*. ISME J 4:862–871

Oberwinkler F, Riess K, Bauer R, Garnica S (2014) Morphology and molecules: the *Sebacinales*, a case study. Mycological Progress 13:445–470

Oliver JD (2005) The viable but nonculturable state in bacteria. J Microbiol 43:93–100

Partida-Martinez LP, Hertweck C (2005) Pathogenic fungus harbours endosymbiotic bacteria for toxin production. Nature 437:884–888

Peškan-Berghöfer T, Shahollari B, Giong PH, Hehl S, Markert C, Blanke V et al (2004) Association of *Piriformospora indica* with *Arabidopsis thaliana* roots represents a novel system to study beneficial plant-microbe interactions and involves early plant protein modifications in the endoplasmic reticulum and at the plasma membrane. Physiol Plant 122:465–477

Piper WE, Ogrodniczuk JS, Joyce AS, McCallum M, Rosie JS, O'Kelly JG et al (1999) Prediction of dropping out in time-limited, interpretive individual psychotherapy. Psyc NET 36:114–122

Qiang X, Weiss M, Kogel KH, Schäfer P (2012a) *Piriformospora indica* - a mutualistic basidiomycete with an exceptionally large plant host range. Mol Plant Pathol 13:508–518

Qiang X, Zechmann B, Reitz MU, Kogel KH, Schäfer P (2012b) The mutualistic fungus *Piriformospora indica* colonizes Arabidopsis roots by inducing an ER stress-triggered caspase-dependent cell death. Plant Cell 24:794–809

Reiss K, Oberwinkler F, Bauer R, Garnicaet S (2014) Communities of endophytic sebacinales associated with roots of herbaceous plants in agricultural and grassland ecosystems are dominated by *Serendipita herbamans* sp. nov. PLoS ONE 9:e94676. doi:10.1371/journal.pone.0094676

Schenk ST, Hernández-Reyes C, Samans B, Stein E, Neumann C, Schikora M, Reichelt M, Mithöfer A, Becker A, Kogel KH, Schikora A (2014) N-Acyl-homoserine lactone primes plants for cell wall reinforcement and induces resistance to bacterial pathogens via the salicylic acid/oxylipin pathway. Plant Cell. doi:10.1105/tpc.114.126763

Schikora A, Schenk ST, Stein E, Molitor A, Zuccaro A, Kogel KH (2011) N-acyl-homoserine lactone confers resistance towards biotrophic and hemibiotrophic pathogens via altered activation of AtMPK6. Plant Physiol 157:1407–1418

Sharma M, Schmid M, Rothballer M, Hause G, Zuccaro A, Imani J et al (2008) Detection and identification of bacteria intimately associated with fungi of the order *Sebacinales*. Cell Microbiol 11:2235–2246

Stein E, Molitor A, Kogel KH, Waller F (2008) Systemic resistance conferred by the root endophyte *Piriformospora indica* to Arabidopsis requires jasmonic acid and the cytoplasmic function of NPR1. Plant Cell Physiol 49:1747–1751

Varma A, Bakshi M, Lou B, Hartmann A, Oelmueller R (2012) *Piriformospora indica*: a novel plant growth-promoting mycorrhizal fungus. Agric Res 1:117–131

Varma A, Verma S, Sudah SN, Franken P (1999) *Piriformospora indica*, a cultivable plant growth-promoting root endophyte. Appl Environ Microbiol 65:2741–2744

Walker V, Bruto M, Bellvert F, Bally R, Muller D, Prigent-Combaret C, Moënne-Loccoz Y, Comte G (2013) Unexpected phytostimulatory behavior for *Escherichia coli* and *Agrobacterium tumefaciens* model strains. Mol Plant-Microbe Interact. 26:495–502

Waller F, Achatz B, Baltruschat H, Fodor J, Becker K, Fischer M et al (2005) The endophytic fungus *Piriformospora indica* reprograms barley to salt-stress tolerance, disease resistance, and higher yield. Proc Natl Acad Sci USA 102:13386–13391

Weiss M, Selosse MA, Rexer KH, Urban A, Oberwinkler F (2011) Sebacinales everywhere: previously overlooked ubiquitous fungal endophytes. PLOS ONE Res 108:1003–1010

Ye W, Shen CH, Lin Y, Chen PJ, Xu X, Oelmüller R et al (2014) Growth promotion-related miRNAs in *Oncidium* orchid roots colonized by the endophytic fungus *Piriformospora indica*. PLoS ONE 9:e84920

Zuccaro A, Lahrmann U, Güldener U, Langen G, Pfiffi S, Biedenkopf D et al (2011) Endophytic life strategies decoded by genome and transcriptome analyses of the mutualistic root symbiont *Piriformospora indica*. PLoS Pathogens 7:e1002290. doi:10.1371/journal.ppat.1002290

Chapter 16
Metabolomics-Mediated Characterization of Endophytic Species in Recalcitrant Tree Species

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Abstract The implication for metabolomics is to capture the endophytic microbes known to promote plant health and overall growth and development. A series of supportable effects on the number of events are mediated and understood by metabolic relations. Current developments in omics world have been made with regard to revealing metabolite release by phyto-microsymbionts, reflecting that they may produce a series of diverse metabolites. These ingredients have a function in resistance and race that may also be required for explicit communication with the plant host. Additionally, a few instances of mutual metabolite manufacturing are recognized and endophytes can moderate a plant–metabolite combination as well. This chapter is focused on a metabolomics tool and understanding the metabolic relations between plants and endophytes. We further discuss the efficient use of helpful plant–microbe interactions in terms of microbial existence in addition to scanning bioactive molecules of commercial interest.

16.1 Introduction

Metabolomics is also defined as the "metabolome," the showcase of the micro level of chemical substrates and compounds in biological systems, with significant investigation into conferring precursors of the metabolism (Hollywood et al.

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2006). According to well-established protocols, metabolomics is one of the latest versions of the "omics" trend and produces significant outcomes that match the omics of gene, transcript, and protein in decoding the molecular repertoires of flora and fauna (Patti et al. 2012). For instance, such functional modulations are performing an incredible role in diverse facets of biological research, from fundamental studies to applied biology (Krug et al. 2012; Holmes et al. 2008)

The evolving systems that can be abridged based on the perception of metabolomics comprise novel opportunities to understand such ecologically persistent signaling molecules. In particular, comparative tools in metabolomics help the identification of compounds that are regulated, although interactive conditions that may play a role, such as pheromones, allelochemicals, induced and activated defenses. Such a method helps to circumvent restrictions of conservative bioassay-dependent assembly elucidation approaches (Umashankar et al. 2015). Moreover, the control of metabolomics is not restricted to the assessment of metabolic profiles of interacting cohorts. Specifically, the connection to other omics techniques assists in disentangling not only the arrays in questions, but all biosynthetic and genetic editing required for an environmental response (Rasmussen et al. 2012).

The majority of recalcitrant plants (i.e., *Eucalyptes* tree spp.) have developed an array of adaptive mechanisms to tackle the limitations of edaphic resources, eventually showing a redundancy of vegetative growth. A detailed study of molecular and metabolic feedback to resource scarcity has been developed with a model (*Arabidopsis thaliana*) system that has the prime focus of sustainability. Such important questions need to be addressed using an evolutionary approach of the biological system in which the genesis of the omic tool is still poorly utilized. Researchers have a free hand approach in terms of the independence of any tool and technique, reflecting that metabolomic approaches are not superiorized, but are rather understood and monitored for the sake of the molecular basis of any systemic function at the plant level.

Like various other plants, tree species have an array of associations with endosymbiotic heterotrophic microbes. A significant example is the relationship between trees such as litchi (*Litchi chinensis* Sonn.), poplar (*Populus*), and bacteria, fungi, mycorrhiza, etc., and this provides an ideal model for studying interactions between flora and microfauna (Graham and Vance 2000, 2003). To enhance this promising area of plant–microbiome interaction and symbiont behavior, the development of mass spectrometry (MS)-based analysis of tree-fiber composition has led to the implementation of metabolomics in recalcitrant plants. Technically, the low digestibility (or degradation rate) of tree cell walls can suppress intake and the chemical composition of the fibers could be implicated in some neglected tree breeding programs (Casler et al. 2008). The measurement of in sacco or in vivo degradation rates of plant material is expensive and not useful for screening individual plants in a breeding program.

This chapter broadly covers the contemporary implications of metabolomics in biochemical, genetic, and systemic niches and confers the prevailing limitations of these new techniques. It elucidates modern expansions in relative metabolomics and postulates the implication of metabolomics in the biological system of organismal connections.

16.2 Endophytic Fungus in the Tree System

Endophytes are integral members of microbiomes, which inherently infect existing plant tissues without triggering any apparent manifestation of an ailment, and exist in mutualistic association with plants for at least part of their life cycle (Biswas et al. 2002; Bacon and White 2000). The term "endophyte" (Greek *endon*, within; *phyton*, plant) was first coined by de Bary (1866). Diverse microorganisms (fungi, bacteria, actinomycetes) have been postulated as endophytes. The predominant endophytes in trees are fungi, which co-exist as a defensive wall and help each other for sustainable adaptation under any natural insults (Staniek et al. 2008).

Various conjectures have been proven in characterizing endophytic fungi, which have been evinced to have significance for the de novo production of numerous bioactive metabolites that may traditionally or inarticulately have been used as therapeutic mediators against numerous diseases (Strobel and Daisy 2003; Strobel 2009; Strobel et al. 2004; Kharwar et al. 2011; Kusari and Spiteller 2011). The environmental niche of endophytic fungi across the host ranges, evolutionary affinity, contamination, establishment, spread patterns, tissue specificity, and symbiotic fitness-related support has been understood for a plethora of plants (Fig. 16.1, Arnold et al. 2003, 2007; Arnold 2005, 2007; Stone et al. 2004; Rodriguez et al. 2009).

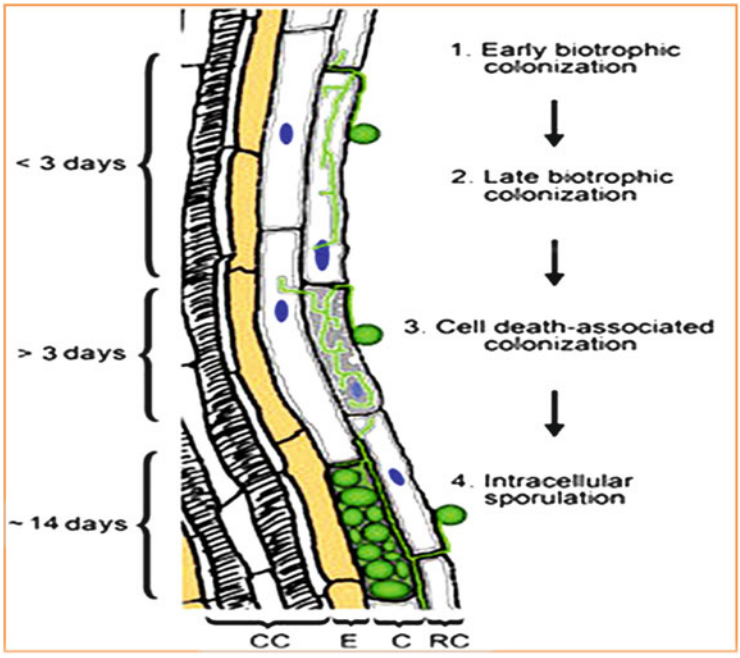

Fig. 16.1 Different stages of root colonization by class 2 endophyte *Piriformospora indica*. *CC* Central cylinder, *C* Cortex, *RC* Rhizodermal cells (reproduced with permission by Rodriguez et al. 2009)

16.3 Metabolomic Profiling for the Beneficial Interaction of Endophytes and Tree Species

Multivariate data mining-based metabolite profiling is a promising approach to categorizing plant–microbe-related samples, revealing candidate precursors of specific metabolites in trees. Such powerful technology is designed to verify the hypothesis that tree plants with and without endophytes have diverse metabolic tags. The profiling approach ensures that the researchers measure metabolites with a combination of the existence of microbes and plant metabolites. Microbial population densities are usually determined, which ranged from 4×106 to 1.2×107 CFU g^{-1} (Christian et al. 2009; Barsch et al. 2006a, b). The corresponding biomass ratio was 1 g of plant material to 1×10^{-5} g of bacteria (Christian et al. 2009). Similar data have revealed the contribution of high-throughput gas chromatography coupled with time-of-flight mass spectrometry (GC-TOF-MS) (Barsch et al. 2006a) to the abundance of microbial biomass in plants, and researchers have measured metabolites fully. The main objective of such a high-throughput metabolomic tool is to track the hidden view of emerging endophytes, such as their diversity, their significance for plant sustainability, and their associations with other organisms (Andrea and Paul 2011).

The present omics world also includes studies that identify the genes and proteins potentially engaged in the plant–microbe interaction. Understanding how the plant–microbe interaction happens allows researchers to ameliorate crop yield and reduce environmental stresses, possibly creating a tree product.

16.4 Metabolomics: Molecular Repertories

The professed terminology "omics" has been implicated in modern biotechnology, broadly aimed at living systems, and plays a significant role in molecular repertoires in numerous ways. Automated methods for analyzing these groups to obtain precise biological information are promising tools that have developed enormously and have enabled the characterization of approaches to larger sets of metabolites. The primary tools considered for this task in metabolomics have recently been reported (Monica and Florencio 2012).

16.5 Metabolomics in the Biochemical Niche of Trees

Tree systems are highly complex systems that need to be understood and analyzed at all levels of intricacy and about which researchers are still beginning to learn. Different molecular levels of proteins and metabolites and their interactions, such

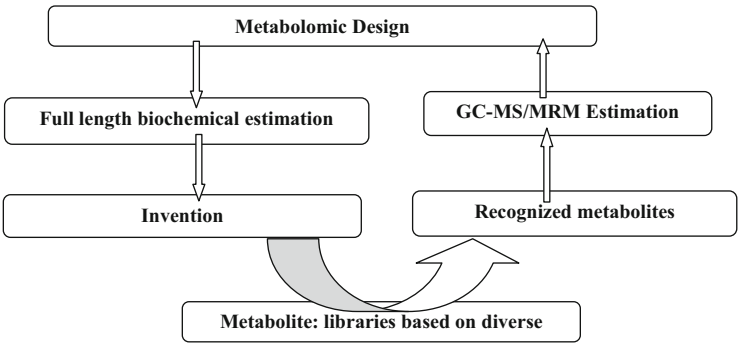

Fig. 16.2 Metabolomic networking system

as gene expression and their underlying pools of biochemistry and physiology, may be noted to constitute a "genotype–phenotype" link (Weckwerth 2003).

Chemical-based study of the environment reveals the natural significance of such products that are known as intermediaries of intra- and inter-organismal interactions. The evolving technologies can be included under the metabolomics thumbprint, which comprises new gateways for exploring these environmentally coherent signaling molecules in tree systems (Constanze and Georg 2015). The specific comparative tools in metabolomics enable the characterization of substrates that are controlled during interlinked mechanisms and that may play a role in either induced or activated defenses. Such an approach enables researchers to overcome problems of the old-fashioned assay-based emphasis on bio-structure. A remarkable strength of metabolomics is that it has been proven to be a promising tool for the comparison of metabolic profiles of interacting substrates. In particular, the link to other omics techniques helps to reveal not only the compounds in question, but the entire biosynthetic and genetic re-wiring required for an ecological response.

In Fig. 16.2 a central strategy is understood at a metabolic level; at the threshold level a semi-quantitative postulation recognizes numerous metabolites, possibly from trees species, in a completely unbiased manner. Reference libraries are constituted based on the tools available, including tree biochemical input and qualitative feedback from innovative phases. Similarly, current libraries are constantly being rationalized with novel substrates from natural tree products or cell wall polymers.

16.6 Conclusion

Until now, the emphasis on metabolomics has been limited. This chapter takes into account future prospects based on existing data. Authors have highlighted the superiority of such soft tools that reveal the tertiary information at a metagenomics

level. Discussion at the metabolite level is linked to the recognized endophytes and their inherent combination with the plant system. Additionally, potential factors are discussed that elucidate the plant–microbe interaction, also describing the screening approaches of bio-active compounds in which metabolomics is prioritized.

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References

Andrea P-A, Paul B (2011) Hidden fungi, emergent properties: endophytes and microbiomes. Annu Rev Phytopathol 49:291–315

Arnold AE (2005) Diversity and ecology of fungal endophytes in tropical forests. In: Deshmukh D (ed) Current trends in mycological research. Oxford & IBH Publishing Co. Pvt. Ltd., New Delhi, India, pp 49–68

Arnold AE (2007) Understanding the diversity of foliar endophytic fungi: progress, challenges, and frontiers. Fungal Biol Rev 21:51–66

Arnold AE, Mejia LC, Kyllo D, Rojas EI, Maynard Z, Robbins N, Herre EA (2003) Fungal endophytes limit pathogen damage in a tropical tree. Proc Natl Acad Sci USA 100:15649–15654

Arnold AE, Henk DA, Eells RA, Lutzoni F, Vilgalys R (2007) Diversity and phylogenetic affinities of foliar fungal endophytes in loblolly pine inferred by culturing and environmental PCR. Mycologia 99:185–206

Bacon CW, White JF (2000) Microbial endophytes. Marcel Dekker Inc., New York

Barsch A, Carvalho HG, Cullimore JV, Niehaus K (2006a) GCMS based metabolite profiling implies three interdependent ways of ammonium assimilation in Medicago truncatula root nodules. J Biotechnol 127:79–83

Barsch A, Tellstrom V, Patschkowski T, Kuster H, Niehaus K (2006b) Metabolite profiles of nodulated alfalfa plants indicate that distinct stages of nodule organogenesis are accompanied by global physiological adaptations. Mol Plant Microbe Interact 19:998–1013

Biswas K, Chattopadhyay I, Banerjee RK, Bandyopadhyay U (2002) Biological activity and medicinal properties of neem (*Azadirachta indica*). Curr Sci 82:1336–1345

Casler MD, Jung HG, Coblentz WK (2008) Clonal selection for lignin and etherified ferulates in three perennial grasses. Crop Sci 48:424–433

Christian S, Kristina U, Dietrich E, Wolfram W (2009) A metabolic aignature of the beneficial interaction of the endophyte Paenibacillus sp. isolate and *in vitro*—grown poplar plants revealed by metabolomics. Mol Plant Microbe Interact 22:1032–1037

Constanze K, Georg P (2015) Metabolomics in chemical ecology. Nat Prod Rep 32:879–1156

De Bary A (1866) Morphologie und Physiologie der Pilze, Flechten, und Myxomyceten. In: Hofmeister's handbook of physiological botany, vol II. Engelmann, Leipzig, Germany

Graham PH, Vance CP (2000) Nitrogen fixation in perspective: an overview of research and extension needs. Field Crops Res 65:93–106

Graham PH, Vance CP (2003) Legumes: importance and constraints to greater use. Plant Physiol 131:872–877

Hollywood K, Brison DR, Goodacre R (2006) Metabolomics: current technologies and future trends. Proteomics 6:4716–4723

Holmes E, Wilson ID, Nicholson JK (2008) Metabolic phenotyping in health and disease. Cell 134:714–717

Kharwar RN, Mishra A, Gond SK, Stierle A, Stierle D (2011) Anticancer compounds derived from fungal endophytes: their importance and future challenges. Nat Prod Rep 28:1208–1228

Krug S, Kastenmuller G, Stuckler F et al (2012) The dynamic range of the human metabolome revealed by challenges. FASEB J 26:2607–2619

Kusari S, Spiteller M (2011) Are we ready for industrial production of bioactive plant secondary metabolites utilizing endophytes? Nat Prod Rep 28:1203–1207

Monica C, Florencio P (2012) Tools for the functional interpretation of metabolomic experiments. Oxford University Press, Oxford, pp. 3–8

Patti GJ, Yanes O, Siuzdak G (2012) Innovation: metabolomics: the apogee of the omics trilogy. Nat Rev Mol Cell Biol 13:263–269

Rasmussen S, Parsons AJ, Jones CS (2012) Metabolomics of forage plants: a review. Ann Bot 110:1281–1290

Rodriguez RJ, White JFJ, Arnold AE, Redman RS (2009) Fungal endophytes: diversity and functional roles. New Phytol 182:314–330

Staniek A, Woerdenbag HJ, Kayser O (2008) Endophytes: exploiting biodiversity for the improvement of natural product-based drug discovery. J Plant Interact 3:75–93

Stone JK, Polishook JD, White JF Jr (2004) Endophytic fungi. In: Mueller G, Bills GF, Foster MS (eds) Biodiversity of fungi: inventory and monitoring methods. Elsevier, Burlington, MA, pp 241–270

Strobel GA (2009) Javanicin, an antibacterial naphthaquinone from anendophytic fungus of neem, *Chloridium* sp. Curr Microbiol 58:233–238

Strobel GA, Daisy B (2003) Bioprospecting for microbial endophytes and their natural products. Microbiol Mol Biol Rev 67:491–502

Strobel GA, Daisy B, Castillo U, Harper J (2004) Natural products from endophytic microorganisms. J Nat Prod 67:257–268

Umashankar T, Govindappa M, Ramachandra YL, Padmalatha Rai S, Channabasava (2015) Isolation and characterization of coumarin isolated from endophyte, alternaria species -1 of Crotalaria pallida and its apoptotic action on HeLa cancer cell line. Metabolomics 5:158. doi:10.4172/2153-0769.1000158

Weckwerth W (2003) Metabolomics in system biology. Annu Rev Plant Biol 54:669–689

Chapter 17
Analytical Techniques to Assess Medicinal Plants Value Addition After Microbial Associations

Priyanka Sharma, Hemesh Joshi, MZ Abdin, Amit C Kharkwal, and Ajit Varma

Abstract In the high-throughput era of science, technologies like high-performance liquid chromatography (HPLC) are growing in importance for their accurate and precise analysis of chemicals and drugs of diverse nature. It is used widely in the pharmaceutical, medicinal, and aromatic plant industry for the isolation and purification of phytomolecules of therapeutic and commercial interest. Due to the gradual increase in the demand for phytochemicals in food and pharmaceutical industries, the importance of process-scale HPLC as a purification tool has been increasing. The most challenging and thrilling field of recent scientific research is the in vitro production of plant secondary metabolites. Therefore, in order to supply required phytochemicals, the in vitro protocol for the establishment of plant culture and production of phytochemicals has to be carefully monitored. In this chapter, the HPLC analysis of secondary metabolite content of an important medicinal plant *Aloe vera* L. has been performed under unstressed and salt-stressed environment. The secondary metabolite content has also been compared upon inoculation of *A. vera* with a symbiotic endophytic fungus *Piriformospora indica*.

17.1 Introduction

Chromatographic techniques are indispensable in ensuring adequate quality in medicinal plants and their preparations. The most widely used techniques are thin-layer (TLC), gas (GC), and high-performance liquid chromatography

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(HPLC), although newer methods, including droplet countercurrent (DCCC) and supercritical fluid (SCFC) chromatography as well as capillary electrophoresis (CE), are finding increased use. The chromatographic methods can be used qualitatively to authenticate the plant or its extracts by producing "fingerprint" chromatograms or "chromatographic profiles." For performing quantitative analysis of plant extracts, HPLC and GC are particularly used. TLC can also be used in combination with spectrophotometric techniques to perform the analysis part (Foster 2001).

17.2 High-Performance Liquid Chromatography

HPLC is a versatile, robust, and widely used technique for the isolation of natural products (Cannell 1998). It is gaining popularity in fingerprint study, for the quality control of herbal plants (Fan et al. 2006). The plant extract usually contains biologically active entity as a minor component. Thus, the resolving power of HPLC is ideally suited to rapid processing of such multicomponent samples on both analytical and preparative scales. Sakakibara et al. (2003) claimed to have found a method capable of quantifying all possible polyphenols in vegetables, fruits, and aromatic beverages including tea. HPLC provides various features, i.e., high resolving power, fast separation, continuous monitoring of column effluent, qualitative and quantitative measurements, isolation, automation of analytical procedures, and data handling. In the pharmaceuticals companies, HPLC is gathering more popularity as a good purification tool. However, the baseline separation using HPLC normally requires complex solvent gradient programs and long analysis times. In addition, unequivocal identification of flavonoids, one of the largest widespread plant secondary metabolite classes, which have similar UV spectra and elution times, cannot be guaranteed. Furthermore, UV detection is unable to consummate the phytochemical task, since lots of plant constituents have no chromophoric groups (Bertoli et al. 2010). The chromatographic separation can remove impurities of different polarities and can reduce the content of an enantiomer in a racemic mixture. In both of these instances, crystallization may be used to prepare the pure product (Gupta and Shanker 2008).

17.3 Chromatography Classification

On the basis of mechanism of separation, chromatography can be classified as *adsorption*, *partition*, *ion exchange*, *size exclusion*, *and affinity chromatography*. In HPLC, separation is mainly governed by adsorption and partition. In adsorption chromatography, separation is based on the difference between the adsorption affinities of the sample components on the surface of an active site, whereas in

partition chromatography, separation is mainly based on the difference between the solubility of sample components in the stationary phase and the mobile phase.

The identification and separation of phytochemicals can be performed using either isocratic system or gradient elution system. The isocratic system uses single mobile phase system, and the composition of the mobile phase remains constant during the elution process. Gradient elution system uses combination of mobile phase systems where the composition of mobile phase changes continuously or stepwise during the elution process. HPLC can also be classified as reverse phase and normal phase chromatography. In reverse phase chromatography, the mobile phase is significantly more polar than stationary phase. Most biomedical substances are separated by reverse phase chromatography using aqueous mixture with methanol, acetonitrile, and additives (buffers, ion pairs). On the other hand, stationary phase is more polar than mobile phase in normal phase chromatography (Gupta and Shanker 2008). Lipophilic substances like oils, fats, and lipids are separated by normal phase chromatography. Commonly used mobile solvents are *n*-hexane, heptane, chloroform, and alcohols.

17.4 Theoretical Aspects of HPLC

Chemical separations can be accomplished using HPLC by utilizing the fact that certain compounds have different migration rates given a particular column and mobile phase. The choice of stationary phase and mobile phase determines the extent or degree of separation. Separation of chemical compounds is carried out by percolation of mobile phase, containing mixture of the components, under gravity through a glass column filled with a finely divided stationary phase. The physical and chemical forces acting between the solute and two phases are responsible for the retention on the chromatographic column. Differences in the magnitude of forces result in the resolution and hence separation of the components (Gupta and Shanker 2008).

Purification of the compound of interest using HPLC is the process of separating or extracting the target compound from other compounds that are structurally similar or contaminants. Each compound should have a characteristic peak under certain chromatographic conditions. Depending on the compound to be separated and how closely related the samples are, conditions such as the proper mobile phase, flow rate, suitable detectors, and columns may be selected to get an optimum separation of the required compound.

17.5 Components of HPLC

An HPLC instrument comprises a solvent delivery pump, a sample introduction device such as an autosampler or a manual injection valve, an analytical column, a guard column, a detector, and a recorder or a printer. The reversed phase columns may be considered the most popular columns used in the analytical separation of plant secondary metabolites, even if new stationary phases have been exploited (Tanaka et al. 2002, 2004). In order to identify any compound by HPLC, a detector must first be selected. Efficiency of the harvesting procedure as well as the accuracy of separation methods relies on the sensitivity of the detector. HPLC system equipped with an autosampler provides a powerful tool to analyze various samples. Once the detector is selected and is set to optimal detection settings, a separation assay must be developed. The parameters of this assay should be such that a clean peak of the known sample is observed from the chromatograph. The identifying peak should have a reasonable retention time and should be well separated from extraneous peaks at the detection levels at which the assay will be performed. UV detectors are popular among all the detectors because they offer high sensitivity (Li et al. 2004) and also because majority of naturally occurring compounds encountered have some UV absorbance at low wavelengths (190–210 nm) (Cannell 1998). Besides UV, other detection methods are also being employed to detect phytochemicals, among which one is the diode array detector (DAD) coupled with mass spectrometer (MS) (Tsao and Deng 2004). Photodiode array detector is generally coupled with HPLC system as it allows to collect spectra in the established wavelength range and to define the spectral homogeneity (purity) of the analytes. This system is one of the most versatile tool in the screening of metabolic profiles of plant extracts (Bertoli et al. 2010).

17.6 Sample Preparation

Processing of the crude material to provide a sample suitable for HPLC analysis as well as the choice of solvent for sample reconstitution can have a significant bearing on the overall success of natural product isolation and identification. The source material, e.g., dried powdered plant, will initially need to be treated to ensure that the compound of interest is efficiently liberated into a solution. In the case of dried plant material, an organic solvent (e.g., methanol, chloroform) may be used as the initial extractant and following a period of maceration, solid material is then removed by decanting off the extract by filtration. The filtrate is then concentrated and injected into HPLC for separation. The usage of guard columns is necessary in the analysis of crude extract. Many natural product materials contain significant level of strongly binding components, such as chlorophyll and other endogenous materials that may in the long term compromise the performance of analytical

columns. Therefore, the guard columns will significantly protect the life span of the analytical columns (Gupta and Shanker 2008).

The most challenging and thrilling field of recent scientific researches is the in vitro production of plant secondary metabolites. There has been a steady rise in the demand for phytochemicals in food and pharmaceutical industries (Bertoli et al. 2010). Therefore, in order to supply required phytochemicals, the in vitro protocol for the establishment of plant culture and production of phytochemical has to be carefully monitored. In this chapter, the HPLC analysis of secondary metabolite content of an important medicinal plant *Aloe vera* L. has been explained under unstressed and salt-stressed environment. The secondary metabolite content has also been compared upon inoculation of *A. vera* with a symbiotic endophytic fungus *Piriformospora indica*.

17.7 Phycobiont: *Aloe vera* L.

For thousands of years, plants have been used as an important source of medicine in pharmaceutical biology. As per WHO estimates, even today, up to 80 % of population still rely on traditional medicines (Yates 2002). Diverse pharmacological activities of medicinal plants have been accredited to phenolic acids and phytochemicals by HPLC, for instance, gallic acid with inflammatory and antibacterial (Kroes et al. 1992; Binutu and Cordell 2000), caffeic acid with anti-inflammatory (Fernandez et al. 1998), ferulic acid with anti-inflammatory and antifungal (Mehrotra 1997), and tannic acid with antioxidant and astringent property (Martha et al. 1983; Khan et al. 2000).

The genus Aloe is a perennial, succulent xerophyte grown in temperate and subtropical parts of the world. *Aloe vera* is cultivated in large quantities because of its high demand in industrial, cosmetics, and pharmaceutical sector. *A. vera* is mostly propagated vegetatively in its natural state, but due to male sterility and low propagation rate, its commercial production and high industry demand are not being met (Natali et al. 1990; Abdi et al. 2013). To catch up with the demand, micropropagation approach for rapid production of this plant has gained importance (Meyer and Staden 1991; Abadi and Kaviani 2010; Marfori and Malasa 2005; Pandhair et al. 2011; Natali et al. 1990; Roy and Sarkar 1991; Abrie and Staden 2001; Corneanu et al. 1994).

The pharmacological actions of *Aloe vera* include anti-inflammatory, anti-arthritic, antibacterial activity, hypoglycaemic effects, and burn healing properties (Choi and Chung 2003). Aloe leaf consists of two parts, each of which produces different substances that have completely different composition and therapeutic properties (Fig. 17.1). The inner parenchymal tissues form a clear, thin, tasteless, jellylike material, called Aloe gel which is used as a moisturizing agent and for the treatment of minor burns, skin abrasions, and irritations (Tarro 1993; Krinsky et al. 2003). The outer pericyclic tubules, occurring just beneath the outer green rind or cutinized epidermis of the leaves, produce bitter yellow exudate, an anthraquinone

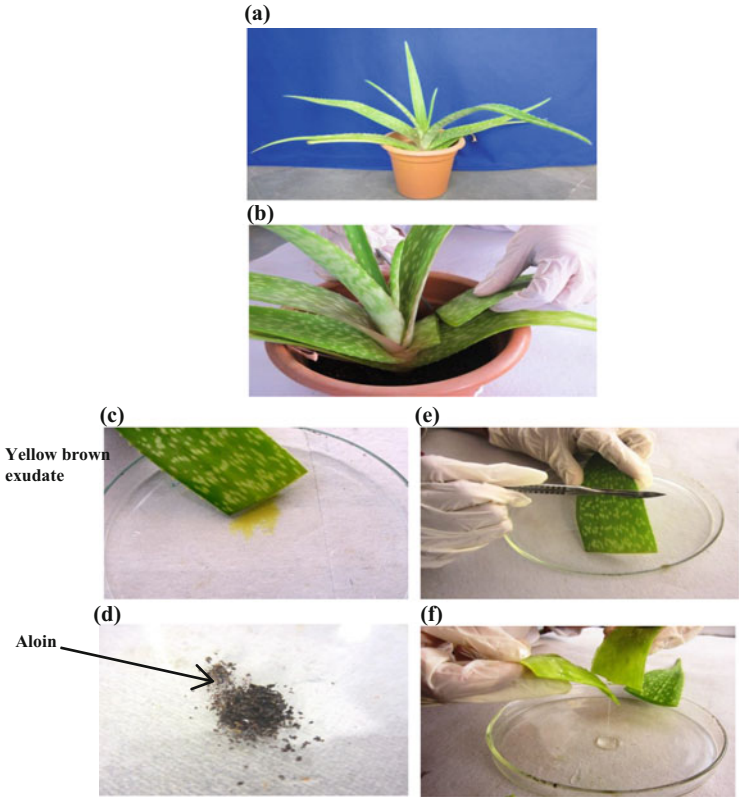

Fig. 17.1 The picture depicts extraction of Aloe gel and anthraquinone "aloin." (**a**) Six-month-old *Aloe vera* plant (**b**) leaf detached with the help of scalpel. Aloe leaf contains two main constituents: (**c**) the *yellow brown* sap exudating from the cut portion and (**d**) upon freeze-drying forms a *dark brown* powder, called aloin, one of the secondary metabolite of *Aloe vera*. Secondly, (**e**, **f**) the Aloe gel, an important constituent of most of the cosmetic products and therapeutics (Sharma et al. 2014b)

that has powerful laxative properties (Vogler and Ernst 1999; Wynn 2005). The presence of aloin, aloe-emodin, and related compounds makes it bitter (Joseph and Raj 2010).

17.8 Mycobiont: *Piriformospora indica*

P. indica is an endophytic fungus of the Sebacinaceae family (Weiß et al. 2004; Verma et al. 1998). It is easily cultivable axenically and colonizes the roots of a wide variety of plant species, in a manner similar to Arbuscular Mycorrhizal (AM) fungi (Varma et al. 1999). The fungus has shown to alter the secondary metabolite content of many plants of medicinal and economic importance. *P. indica*

facilitates nutrient uptake in both monocot and dicot plants. It enhances the growth and overall biomass (Yadav et al. 2010; Varma et al. 2000), induces early flowering (Das et al. 2012a), increases resistance against a number of pathogens, and allows the plant to survive under stressed environment (Das et al. 2012b; Harman 2011). Various factors responsible for plant growth stimulation have also been determined using HPLC.

One g aliquot of freshly harvested *P. indica* was washed with sterile distilled water and subsequently treated with 5 ml of 80 % aqueous methanol. The solution was centrifuged at 6000 g for 15 min, and the supernatant was used as sample for HPLC analysis. The analysis showed seven peaks in the hyphal extract and one main peak in the culture filtrate. A major peak in the preparative HPLC analysis of hyphal extract and culture filtrate was identified as benzoic acid. The function of this compound is not yet clear. However, compounds identical to benzoic acid and its analogues (benzoic acid, a-hydroxybenzoic acid, 3-4 di-hydroxybenzoic acid, vanillic acid, cinnamic acid, p-coumaric acid, caffeic acid, ferulic acid) did not show any stimulation on the plants tested (Varma et al. 2001). The same method as described above was used to A cyclohexomone derivative (blumenin) which is accumulated in roots of cereals, and other members of Poaceae colonized by arbuscular mycorrhizal fungi (Maier et al. 1995; Fester et al. 1998) were detected. The exact function of cyclohexomones' accumulation is not yet known. It is speculated that these secondary compounds might be involved in the regulation of mycorrhizal colonization (Fester et al. 1998). HPLC analysis of methanolic extracts from 6-week-old plant roots (barley, maize, and foxtail millet *Setaria italica*), treated and non-treated with *P.indica*, showed quantitative but nonqualitative changes. There were no changes recorded in case of rice and wheat. The UV spectra obtained from HPLC photodiode array detector showed a cluster of peaks between 7.5 and 12.5 min of retention time for extracts from maize, barely, rice, and foxtail millet co-inoculated with *P. indica*, indicating the presence of indole derivatives, e.g., tryptophan, tryosine, and tyramine or their derivatives (unpublished results). Root extracts of maize showed the presence of cyclic hydroxamine acids like DIMBOA (2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one) in wheat but not in rice, barley, and Setaria. HPLC analysis of methanolic extracts of infected maize roots showed eight different peaks with a photodiode array detector with typical UV spectra of benzoxazinone derivatives. The chemical structure of these compounds needs to be identified.

17.9 Assessment of Plant Secondary Metabolite

Anthraquinone derivatives and polysaccharides in *Aloe vera* gel have various therapeutic properties and are used for wound healing, radiation damage repair, etc. (Ammar et al. 2010). Aloin, one of the major anthraquinone derivative and secondary metabolite in the leaf of *A. vera*, was quantified using HPLC.

Sample Preparation for HPLC
Sample extraction protocol of Kispotta et al. (2012) was followed. Fresh leaves of *A. vera* plantlets were harvested and cuts were made on the leaf. The brown exudate from the leaf was collected. It was freeze--dried and extracted with ethanol for 1 h at 50 °C using sonicator and centrifuged at 3000 g for 10 min. The supernatant was further filtered using 22 μm filter.

Standard Curve
Different concentrations of standard of Aloin, ranging from 0.1–0.6 mg/ml, were taken in triplicates. These stock solutions were stored at −20 °C. Calibration curve was obtained by plotting the peak area against concentrations of standard solution, and they showed linear relationship.

Detection and Quantification of Aloin
The aloin content was determined by HPLC analysis using Waters system, which consisted of dual wavelength UV absorbance detector (Waters 2487), binary HPLC pump (Waters 1525), and Waters temperature control system. The separation was carried out on a C18 column. Analysis was performed at a flow rate of 1 ml/min. Two different mobile phase systems consisting of TFA in MQ water and HPLC grade methanol were used and gradient system was followed (Kispotta et al. 2012). The filtrate obtained was subjected to HPLC analysis (injection volume—20 μl). The monitoring was done at wavelength of 293 nm.

HPLC Validation
The precision and accuracy of the method were performed through within-day and between-day run validations. The within-day precision and accuracy were determined for each standard on three concentrations with five replicates on a single day. Phenolic compound was identified by comparing the retention time with that of standard compound. Quantity of aloin produced was calculated based on the area of sample peak, concentration, and peak area of standard.

17.10 Effect of *P. indica* Inoculation on Aloin Content of *A. vera* Plantlets

The *A. vera* plantlets were cocultivated with the fungus *P. indica* in vitro by the protocol given by Sharma and colleagues (2014a). Aloin content in *P. indica* inoculated (Treated) and non-inoculated (Control) *A. vera* plantlets was compared, and it was found to be 1.28 ± 0.057 (mean ± SE) mg aloin/g of leaf exudate for *P. indica* inoculated plantlets and 0.844 ± 0.01 for the non-inoculated ones, marking an overall increase of 52.53 % (Fig. 17.2). Each dataset represents an average of six replicates.

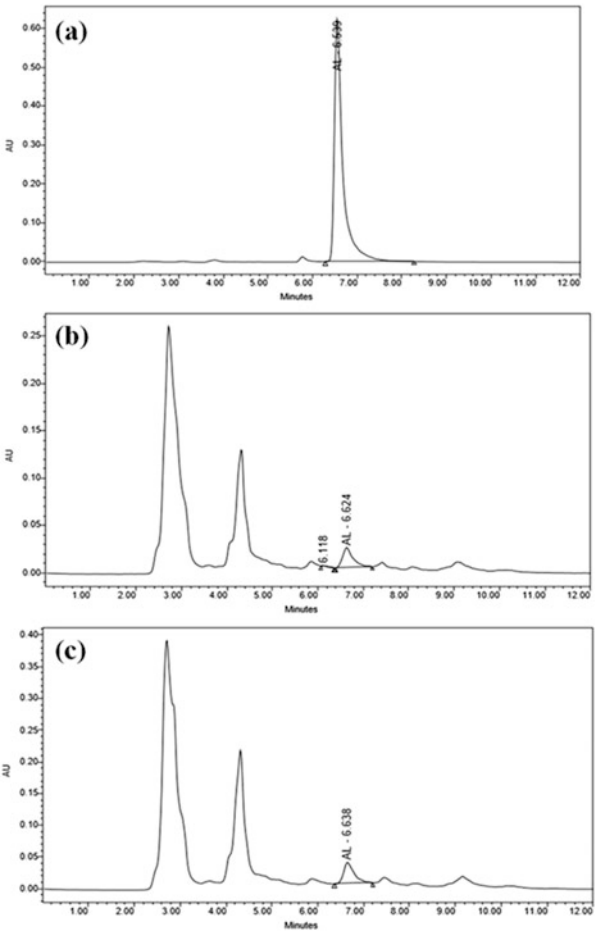

Fig. 17.2 Effect of *P. indica* on aloin content of *Aloe vera* plantlets: (**a**) standard aloin, (**b**) control plants, and (**c**) plants treated with *P. indica*

17.11 Effect of Salt Treatment on Aloin Content of *P. indica* Inoculated and Non-inoculated Plantlets

Aloin content of *P. indica* inoculated and non-inoculated *A. vera* plantlets was also determined after giving 2 months of salt stress. Two levels of fungal inoculation (non-inoculated plantlets and plantlets inoculated with *P. indica*) and four levels of salt (0, 100, 200, and 300 mM NaCl) were the treatments ($2 \times 4 = 8$).

The aloin content as determined by HPLC was found to be 2.67 ± 0.25 mg aloin/g of leaf exudate for *P. indica* inoculated plantlets and 2.43 ± 0.3 for the non-inoculated ones, marking an increase of 9.87 %, which is nonsignificant at $P < 0.05$. The aloin content increased with increase in salt stress. Aloin content in the treated plantlets increased between 43.8 % and 27.32 % with increase in salt stress from 100 mM to 200 mM, respectively. At 300 mM, the inoculated plantlets had

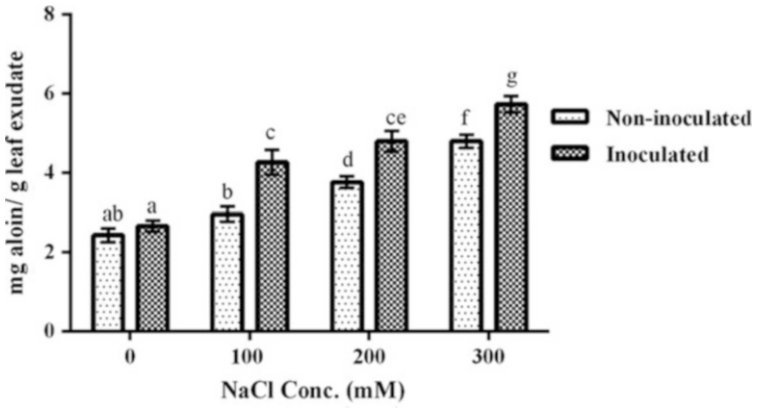

Fig. 17.3 Effect of *P. indica* inoculation (inoculated and non-inocualted) and salt treatment (0, 100, 200, and 300 mM) on Aloin content of *Aloe vera*. Each dataset represents an average of three replicates. The error bars represent SE. Different letters on the *bar* indicate that the values differ significantly at $P < 0.05$ as determined by analysis of variance

5.73 ± 0.35, in comparison with 4.8 ± 0.3 of non-inoculated plantlets, which was 51.9 % higher (Fig. 17.3). Each dataset represents an average of three replicates.

17.12 Conclusion

Chromatograms of plant extracts are used as fingerprints and compared with standard compounds in order to identify the plant material and its constituents. HPLC is thus one of the best suited technique for an efficient separation of the crude plant extracts. Its significance will continue to grow because of the increasing requirements for high-purity molecules.

Statistical Analysis
One-way ANOVA with Dunnett's posttest was performed using GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego, California, USA, www.graphpad.com. Where applicable, the results were expressed as mean ± standard error (SE).

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References

Abadi DH, Kaviani B (2010) *In vitro* proliferation of an important medicinal plant Aloe – a method for rapid production. Aust J Crop Sci 4:216–222

Abdi G, Hedayat M, Modarresi M (2013) *In vitro* micro propagation of *Aloe vera* – impacts of plant growth regulators, media and type of explants. J Biol Environ Sci 7:19–24

Abrie AL, Staden JV (2001) Micro propagation of the endangered *Aloe polyphylla*. Plant Growth Regul 33:19–23

Ammar NM, Singab ANB, Ahmady SH, Anssary AA, Haggag EG, Shabban RS (2010) Phytochemical and biological studies of some polysaccharides isolated from Aloe, Tamarindus. Opuntia and citrus. J Arab Soc Med Res 5:141–152

Bertoli A, Ruffoni B, Pistelli L, Pistelli L (2010) Analytical methods for the extraction and identification of secondary metabolite production in 'in vitro' plant cell cultures. Adv Exp Med Biol 698:250–266

Binutu OA, Cordell GA (2000) Gallic acid derivatives from Mezoneuron benthamianum leaves. Pharm Biol 34:284–286

Cannell RJP (1998) Natural products isolation. Human Press, Totowa, pp. 165–208

Choi S, Chung MH (2003) A review on the relationship between *Aloe vera* components and their biologic effects. Semin Integr Med 1:53–62

Corneanu M, Corneanu G, Vekas M, Minea R (1994) *In vitro* organogenesis of *Aloe arborescencs* (Liliaceae). Rev Roum Biol 39:45–52

Das A, Kamal S, Shakil NA, Sherameti I, Oelmuller R, Dua M, Tuteja N, Johri AK, Varma A (2012a) The root endophyte fungus *Piriformospora indica* leads to early flowering, higher biomass and altered secondary metabolites of the medicinal plant, *Coleus forskohlii*. Plant Signal Behav 7:1–10

Das A, Sheramati I, Varma A (2012b) Contaminated soils: physical, chemical and biological components. In: Varma A, Kothe E (eds) Bio-geo interactions in metal-contaminated soils. Springer, Heidelberg, pp 1–16

Fan XH, Cheng YY, Ye ZL, Lin RC, Qian ZZ (2006) Multiple chromatographic fingerprinting and its application to the quality control of herbal medicines. Anal Chim Acta 555:217–224

Fernandez MA, Saenz MT, Garcia MD (1998) Anti inflammatory activity in rats and mice of phenolic acids isolated from Scrophularia frutescens. J Pharm Pharmacol 50:1183–1186

Fester T, Maier W, Strack D (1998) Accumulation of secondary compounds in barley and wheat roots in response to inoculation with arbuscular mycorrhizal fungi and co-inoculation with rhizosphere bacteria. Mycorrhiza 8:241–246

Foster A (2001) The quality control of herbal medicinal products. In: Sandberg F, Corrigan D (eds) Natural remedies: their origins and uses. Taylor and Francis, London, pp 11–21

Gupta MM, Shanker K (2008) Process-scale high performance liquid chromatography for medicinal and aromatic plants. In: Khanda SS, Singh Khanuja SP, Longo G, Rakesh DD, Khanda SS, Singh Khanuja SP, Longo G, Rakesh DD (eds) Extraction technologies for medicinal and aromatic plants. International Center for Science and High Technology, Trieste, Italy, p 260

Harman GE (2011) Multifunctional fungal plant symbiont: new tools to enhance plant growth and productivity. New Phytol 189:647–649

Joseph B, Raj SJ (2010) Pharmacognostic and phytochemical properties of *Aloe vera* linn–an overview. Int J Pharm Sci Rev Res 4:106–110

Khan NS, Ahmed A, Hadi SN (2000) Antioxidant, pro-oxidant properties of tannic acid and its binding to DNA. Chem Biol Interact 125:177–189

Kispotta A, Srivastava MK, Dutta M (2012) Free radical scavenging activity of ethanolic extracts and determination of aloin from *Aloe vera* L. leaf extract. Int J Med Arom Plants 2:612–618

Krinsky DL, Hawkins EB, Pelton R, Willis NA, Lavalle JB (2003) Natural therapeutics pocket guide, 2nd edn. Lexi-Comp, Cleveland, p. 379

Kroes BH, Van den Berg AJJ, Quarles Van Offord HC, Van Dijk H, Labodie RP (1992) Anti-inflammatory activity of gallic acid. Planta Med 58:499–503

Li HB, Jiang Y, Chen F (2004) Separation methods used for Scutellariabaicalensis active components. J Chromatogr B 812:277–290

Maier W, Peipp H, Schmidt J, Wray V, Strack D (1995) Level of a terpenoid glycoside (blumenin) and cell wall bound phenolics in some cereal mycorrhizal. Plant Physiol 109:465–470

Marfori EC, Malasa AB (2005) Tissue culture for the rapid clonal propagation of *Aloe barbadensis* Miller Philipp. Agric Sci 88:167–170

Martha, W, Susan, B, Rosemary, FB, Elizabeth, SO (1983) The Merck index, 10th edn. Merc, Rathway, pp 4218, 2268, 6784, 8189

Mehrotra RS (1997) Defense mechanism in plants. In: Walker JC (ed) Plant pathology. Tata-McGrew Hil, New Delhi, p 544

Meyer HJ, Staden JV (1991) Rapid *in vitro* propagation of *Aloe barbadensis* Mill. Plant Cell Tissue Organ Cult 26:167–171

Natali L, Sanchez IC, Cavallini A (1990) *In vitro* culture of *Aloe barbadensis* Mill: micro propagation from vegetative meristems. Plant Cell Tissue Organ Cult 20:71–74

Pandhair V, Diviya J, Joginder S, Gosal SS (2011) Biochemical studies of *Aloe vera* (*Aloe barbadensis* Miller) gel of the field grown and micro propagated plants at different developmental stages. J Plant Biochem Biotechnol 20:283–287

Roy SC, Sarkar A (1991) *In vitro* regeneration and micro propagation of *Aloe vera*. Sci Hortic 47:107–114

Sakakibara H, Honda Y, Nakagawa S, Ashida H, Kanazawa K (2003) Simultaneous determination of all polyphenols in vegetables, fruits and teas. J Agric Food Chem 51:571–581

Sharma P, Kharkwal AC, Abdin MZ, Varma A (2014a) *Piriformospora indica* improves micro propagation, growth and phyto chemical content of *Aloe vera* L. Plants. Symbiosis 64:11–23

Sharma P, Kharkwal AC, Kharkwal H, Abdin MZ, Varma A (2014b) A review on pharmacological properties of *Aloe vera*. Int J Pharm Sci Rev Res 29:31–37

Tanaka N, Kobayashi H, Ishizuka N (2002) Monolithic silica columns for high efficiency chromatographic separations. J Chromatogr 965:35–49

Tanaka N, Kimura H, Tokuda D, Hosoya K, Ikegami T, Ishizuka N, Minakuchi H, Nakanishi K, Shintani Y, Furuno M, Cabrera K (2004) Simple and comprehensive two dimensional reversed phase HPLC using monolithic silica columns. Anal Chem 76:1273–1281

Tarro, V.E., (1993). The honest herbal: a sensible guide to the use of herbs and related remedies 3rdrd ed., Pharmaceutical Products Press, New York, pp 25–28

Tsao R, Deng Z (2004) Separation procedures for naturally occurring antioxidant phytochemicals. J Chromatogr B 812:85–99

Varma A, Verma S, Sudha, Sahay N, Buttehorn B, Franken P (1999) *Piriformospora indica*, a cultivable plant growth promoting root endophyte. Appl Environ Microbiol 65:2741–2744

Varma A, Singh A, Sudha, Sahay N, Sharma J, Roy A, Kumari M, Rana D, Thakran S, Deka D, Bharti K, Hurek T, Blechert O, Rexer KH, Kost G, Hahn A, Hock B, Maier W, Walter M, Strack D, Kranner I (2000) Mycota IX, vol 8. Springer, New York, pp. 225–253

Varma A, Singh A, Sudha S, Sharma J, Roy A, Kumari M, Rana D, Thakran S, Deka D, Bharti K, Hurek T, Blechert O, Rexer KH, Kost G, Hahn A, Maier W, Walter M, Strack D, Kranner I (2001) *Piriformosporaindica* – an axenicallyculturable mycorrhiza-like endosymbiotic fungus. In: HOCK B (ed) Mycota IX. Springer, Berlin, pp 123–150

Verma S, Varma A, Rexer K, Hassel A, Kost G, Sarbhoy A, Bisen P, Butehorn B, Franken P (1998) *Piriformospora indica*, gen. et sp. nov., a new root-colonizing fungus. Mycologia 90:896–903

Vogler B, Ernst E (1999) *Aloe vera*: a systematic review of its clinical effectiveness. Br J Gen Pract 49:823–828

Weiß M, Selosse MA, Rexer KH, Urban A, Oberwinkler F (2004) Sebacinales: a hitherto overlooked cosm of heterobasidiomycetes with a broad mycorrhizal potential. Mycol Res 108:1002–1010

Wynn RL (2005) *Aloe vera* gel: update for dentistry. Pharmacol Today: Gen Dent 1:6–9

Yadav V, Kumar M, Deep DK, Kumar H, Sharma R, Tripathi T, Tuteja N, Saxena AK, Johri AK (2010) A phosphate transporter from the root endophytic fungus *Piriformospora indica* plays a role in phosphate transport to the host plant. J Biol Chem 285:26532–26544

Yates A (2002) Yates garden guide. HarperCollins, Moss Vale

Chapter 18
Omics: Tools for Assessing Environmental Microbial Diversity and Composition

Manoj Kumar, Neeraj Shrivastava, Priyanku Teotia, Pankaj Goyal, Ajit Varma, Shivesh Sharma, Narendra Tuteja, and Vivek Kumar

Abstract Conventional culture-based techniques are imperative in investigating the microbial ecology of natural and anthropogenically impacted environments, but they are tremendously prejudiced and unfair in their evaluation of microbial genetic assortment by selecting a specific inhabitant of microbes. Due to current progresses in genomics and sequencing methodologies, microbial community studies using culture nondependent molecular procedures have begun a new epoch of microbial ecology. Molecular studies of ecological communities have discovered that cultivable microbial segment represents <1 % of whole number of prokaryotic species existing in any sample. Various molecular approaches based on direct isolation and analysis of genetic material, proteins, and lipids from ecological samples have been discovered and shown the structural and functional information about microbial groups. Novel molecular tactics such as genetic fingerprinting, metagenomics, metaproteomics, metatranscriptomics, and proteogenomics are imperative and essential for determining and describing the huge microbial variety along with understanding of their synergistic behavior with biotic and abiotic ecological factors. This chapter recapitulates the latest development in molecular microbial ecology area paying attention to new methods and tactics that suggest novel understandings into phylogenetic, practical, and functional assortment of microbial assemblages. The benefits and drawbacks of normally employed molecular techniques to investigate microbial structures are also discussed along with probable applications of novel molecular approaches and how they can provide a outlook on developing technologies for environmental microbial community profiling.

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18.1 Introduction

Soil microbial characteristics are being assessed progressively and precisely as a delicate and complex indicator of soil health because of relationships between microbiome, soil and plant quality, and environment sustainability (Barberan et al. 2012). Understanding of microbial characteristics such as biomass, activity, communication, and diversity is significant to scientists in advancing information and data contributing to overall soil health. Analysis of these results might be valuable to farmers, scientists, and extension personnel in planning and developing practical measures of soil quality. Soil microbial characteristic studies have been frequently conducted at the process level, where biomass, respiration rates, quorum sensing, and enzyme activities have been studied with reference to organism- or community-level responses to the changes in soil properties. Though these process-level studies provide an imperative knowledge of all microbial processes and their possible role in soil health. Studies also tell us something about qualitative community-level changes because any given microbial process may be carried out by dissimilar taxa. Additionally, these process-level measurements are limited in their ability to define a specific microbial ecosystem (Myrold et al. 2013).

Society-level microbial synergism is multifaceted and intricate, with discrete species relying on the occurrence, role, communication, and interaction with numerous other species. Consequently, measurable and qualitative changes in the configuration and arrangement of soil microbial communities may serve as significant and delicate indicators of both short- and long-term changes in soil health. The investigation of soil microbial structure should involve not only determination of microbial biomass and diversity but also evaluation of microbial growth, distribution, function, and the nature of communication among various species (Cong et al. 2015).

The long-lasting challenges in soil microbial ecology have been the development of applicable methods to (a) conclude and establish which microorganisms are present in soil and (b) and to determine microbial function in situ. These challenges have been aggravated by the problems and hitches of microbial separation from the soil matrix and from plant part, the morphological similarities among many microbes found in soils, and changing microbial taxonomies. Additionally, the small size of soil microbial communities has made direct picturing more problematic than with macro-organisms (Faust et al. 2015).

Over the past ten years, the approach to know and identify the soil microbial structure and their relationship has changed vividly. Plentiful novel techniques and methods are now accessible, which allow soil microbiologists to get access to more of the microorganisms residing in soil and allowing for better assessments of microbial diversity. In this chapter, we concisely consider some of the vital approaches for studying soil microbial structures. Our aim is to discuss the novel culture-independent or omics techniques in perception with the conventional culture-based methods for evaluating microbial assortment.

18.2 Reasons for Studying Microbial Diversity

Within natural microbial populations, a large amount of genetic information is "waiting" to be discovered. It has been recorded that culturable bacteria represent a minor fraction of the total bacterial population present (Fakruddin and Mannan 2013). However, it is important to continue the work on both the culturable and the nonculturable bacteria from different environments. Diversity studies are also important for comparison between samples. Another essential reason for studying the microbial diversity is the lack of sufficient knowledge about the existing and extinct microorganisms. There is no agreement among scientists that how many species exist in the world, the potential usefulness of most of them, or the rate at which they are disappearing or emerging. The capability of an ecosystem to resist severe perturbations or stress conditions can partly be dependent on the diversity within the system, and therefore, diversity analyses are vital and important in order to:

- Upsurge the knowledge of the diversity of genetic resources and comprehend the microorganism's distribution.
- Upsurge the knowledge of the practical and operative role of diversity.
- Categorize and detect differences in diversity associated with management disturbing.
- Recognize and identify the biodiversity regulation and management.
- Know the biodiversity importance and consequences.

18.3 Factors Governing Microbial Diversity

In a bacterial community, numerous different organisms will achieve same processes and perhaps be present in same niches (Delmont et al. 2015). Main dynamics that influences microbial diversity could be classified into two major groups, i.e., abiotic and biotic factors. Abiotic factors include both physical and chemical or physicochemical factors such as availability of water, oxic/anoxic conditions, salinity, pressure, temperature, pH, organic pollutants, heavy metals, chemical fertilizers, pesticides, allelopathy factors, antibiotics, etc. (Allen and Banfield 2005). Generally, all environmental variations affect in dissimilar ways and to diverse degrees, resulting in a swing in diversity profile. Biotic factors include other live microbes, plasmids, phages, cell-free nucleic acids and proteins, transposons that are types of accessory DNA that influence the genetic properties, and in most cases, the phenotypes of their host and therefore have a huge impact on microbial diversity (Zhao et al. 2012). Furthermore, several protozoa do influence the microbial diversity, as they feed on them (Rønn et al. 2002; Paisie et al. 2014).

18.4 Culture Techniques in Eco-microbiology: Applications and Restraints

The standard culture techniques to enrich, isolate, and characterize the microbial community are by employing commonly used growth media such as Nutrient Agar, Luria-Bertani medium, Potato Dextrose Agar, and Tryptic Soy Agar (Kumar 2011). The chief restraint of conventional culture-based methods is that more than 99 % of the microbes in any soil ecosystem observed through a microscope are not cultivable by standard culturing techniques (Hugenholtz 2002). Several improved cultivation procedures and culture media have been devised that mimic natural environments in terms of nutrients (composition and concentration), oxygen gradient, pH, etc., to maximize the cultivable fraction of microbial communities. In this regard, a technique has been devised for culturing the uncultured microorganisms from diverse environments including deep seawater, marine sediments, and unexplored soil environment that involved encapsulation of cells in gel microdroplets for large-scale microbial cultivation under low nutrient flux conditions (Zengler et al. 2005). Nonetheless, not all "uncultured" organisms are cultivable, and many of them remain "unculturable." These organisms, although viable in their natural environments, do not grow under laboratory conditions and remain in a "viable but nonculturable" (VBNC) stage (Vieira and Nahas 2005). Such VBNC organisms could represent completely novel groups and may be abundant or very active but remain untapped by standard culture methods.

Molecular microbial surveys based on 16S rRNA genes reveal that candidate bacterial divisions such as BRC1, OP10, OP11, SC3, TM7, WS2, and WS3 have no cultured representatives and are known only by their molecular sequences (Schloss and Handelsman 2004). These division-level clades, such as OP11, are highly diverse and widely distributed in different environments and are considered as "candidate divisions" to reflect our limited knowledge due to the lack of any cultured representative. Studies suggest the existence of at least 50 bacterial phyla with half represented entirely by molecular sequences (Kumar et al. 2016). Additionally, microorganisms retrieved using common culture methods are rarely numerically abundant or functionally significant in the environment from which they were cultured. These cultured microorganisms are considered as the "weeds" of the microbial world and constitute <1 % of all microbial species (Streit and Schmitz 2004). For example, most of the isolates cultured from soil samples belong to one of four phyla (the "big four"), Proteobacteria, Firmicutes, Bacteroidetes, and Actinobacteria, primarily due to their ease of cultivation under laboratory conditions. Although Acidobacteria constitutes on average 20 % of soil bacterial communities, these organisms are difficult to culture and are represented by few genera (Schloss and Handelsman 2004). These findings suggest that molecular techniques that circumvent the need for isolation and cultivation are highly desirable for in-depth characterization of environmental microbial communities.

18.5 Omics Technology

Metagenomics is the investigation of collective microbial genomes retrieved directly from environmental samples and does not rely on cultivation or prior knowledge of the microbial communities (Riesenfeld et al. 2004). Metagenomics is also known by other names such as environmental genomics or community genomics, or microbial ecogenomics. Essentially, metagenomics does not include methods that interrogate only PCR-amplified selected genes (e.g., genetic fingerprinting techniques) as they do not provide information on genetic diversity beyond the genes that are being amplified. In principle, metagenomic techniques are based on the concept that the entire genetic composition of environmental microbial communities could be sequenced and analyzed in the same way as sequencing a whole genome of a pure bacterial culture as discussed in the preceding section. Metagenomic investigations have been conducted in several environments such as soil, the phyllosphere, the ocean, and acid mine drainage and have provided access to phylogenetic and functional diversity of uncultured microorganisms (Rastogi et al. 2010). Thus, metagenomics is crucial for understanding the biochemical roles of uncultured microorganisms and their interaction with other biotic and abiotic factors. Environmental metagenomic libraries have proved to be great resources for new microbial enzymes and antibiotics with potential applications in biotechnology, medicine, and industry (Riesenfeld et al. 2004; Singh et al. 2009).

Metagenomic library construction involves the following steps: (1) isolation of total DNA from an environmental sample, (2) shotgun cloning of random DNA fragments into a suitable vector, and (3) transforming the clones into a host bacterium and screening for positive clones. Metagenomic libraries containing small DNA fragments in the range of 2–3 kb provide better coverage of the metagenome of an environment than those with larger fragments. It has been estimated that to retrieve the genomes from rare members of microbial communities at least 1011 genomic clones would be required (Smith and Osborn 2009). Small-insert DNA libraries are also useful to screen for phenotypes that are encoded by single genes and for reconstructing the metagenomes for genotypic analysis. Large-fragment metagenomic libraries (100–200 kb) are desirable while investigating multigene biochemical pathways. Metagenomic libraries could be screened either by sequence-driven metagenomic analysis that involves massive high-throughput sequencing or by functional screening of expressed phenotypes. Sequence-driven massive whole-genome metagenomic sequencing sheds light on many important genomic features such as redundancy of functions in a community, genomic organizations, and traits that are acquired from distinctly related taxa through horizontal gene transfers (Koonin et al. 2001; Werner 2014).

In function-driven metagenomic analysis (functional metagenomics), libraries are screened based on the expression of a selected phenotype on a specific medium (Fig. 18.1). A wide variety of biochemical activities have been discovered in environmental metagenomic libraries. For example, novel antibiotics (e.g., turbomycin, terragine), microbial enzymes (e.g., cellulases, lipases, amylases),

and proteins (e.g., antiporters) have been identified in soil metagenomic libraries (Rondon et al. 2000; Bergmann et al. 2014). Function-driven metagenomic approaches require successful expression of a desired gene in a heterologous host such as *E. coli*. Thus, a major limitation is very low level or no expression of the majority of environmental genes in *E. coli*. In some cases, improved gene expression can be achieved by transforming metagenomic DNA into several additional surrogate hosts such as *Bacillus, Streptomyces*, *Pseudomonas*, and *Agrobacterium* (Fig. 18.1). Strategies that can enhance heterologous expression of unknown genes in host cells are highly desirable. For example, genetically engineered *E. coli* that can support the translation and transcription of a wide diversity of genes or cloning vectors with strong promoters that can provide additional transcription factors will be highly desirable. In a metagenomic library, the frequency of active gene clones expressing a phenotype is typically very low. For example, in an environmental metagenomic library established from soil, only one in 730,000 clones showed lipolytic activity (Henne et al. 2000). The DNA and inferred protein sequence of a novel lipolytic clone exhibited only a moderate identity (<50 %) with known lipases, indicating that it could be from an uncultured organism. Low occurrence of actively expressing clones in metagenomics libraries necessitates improved high-throughput screening and detection assays (Pearson 2013). Keeping the above facts in mind, Fig. 18.1 describes the isolation of DNA from soil samples, library construction, cloning, mining, and their potential application in biotechnology.

18.6 Proteogenomics

In metaproteomics, protein sequences could be identified with confidence only if they have significant homology to existing proteins in available databases. However, in most of the environmental proteomic surveys, proteins are only distantly related to known database sequences. Therefore, it appears that the majority of short protein sequences retrieved from metaproteomes will remain unidentified and cannot be assigned to their functional and phylogenetic features (Hettich et al. 2013). However, these limitations have been overcome by combining the metaproteomic and metagenomic approaches together under the name of "proteogenomics" (Banfield et al. 2005). In community proteogenomics, total DNA and proteins are extracted from the same sample, which allows linking of biological functions to phylogenetic identity with greater confidence. The metagenomic part of the proteogenomic approach plays a very significant role and increases the identification of protein sequences by metagenomic analysis of the same sample from which the proteins were extracted (Johnson-Rollings et al. 2014).

The proteogenomics approach was applied to decipher phyllosphere bacterial communities in a study by Delmotte et al. (2009). Bacterial biomass was harvested from leaf surfaces of soybean, clover, and *Arabidopsis*, and proteins were extracted.

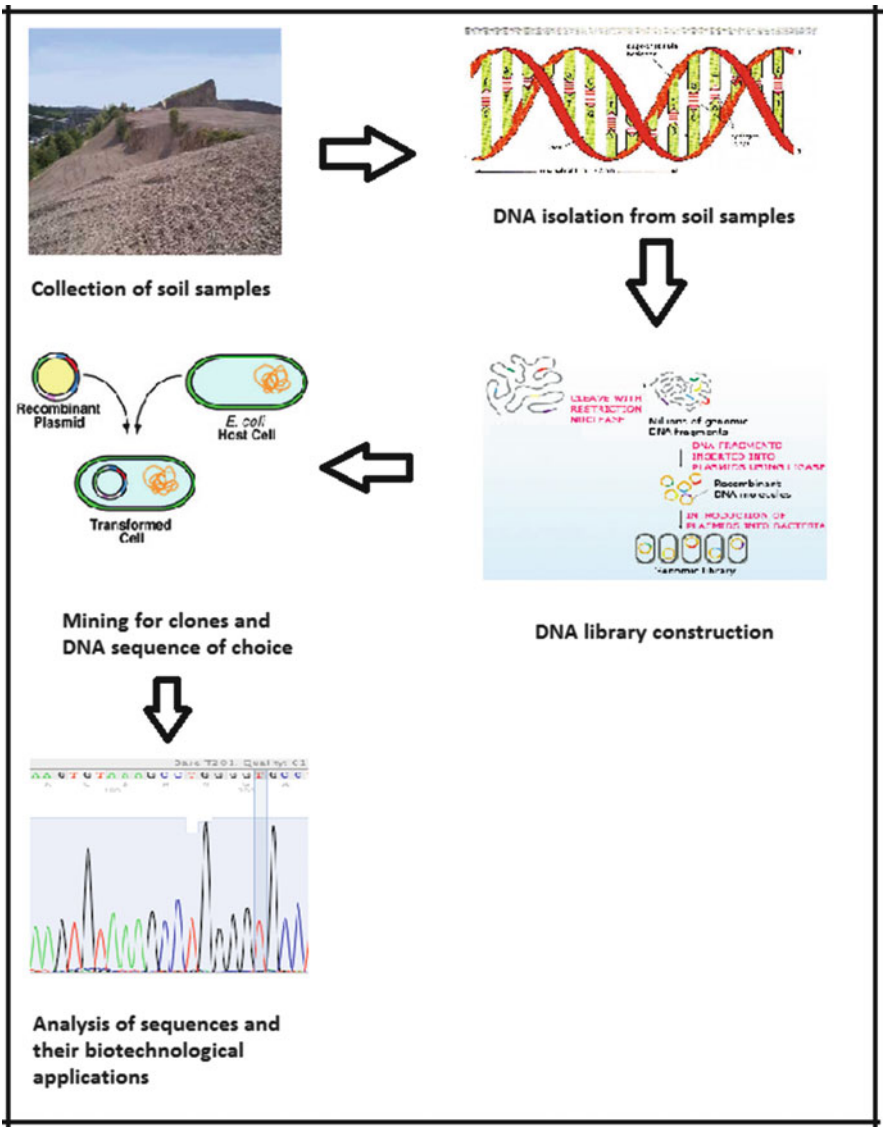

Fig. 18.1 Metagenomic approach for DNA isolation from environment

This was followed by tryptic digestion and separation of fragments by liquid chromatography and analysis by mass spectrometry. This led to the identification of 2883 unique proteins from nearly one-half million spectra. The metagenomic data generated from the DNA extracted from the same pool of bacterial biomass significantly increased (up to 74 %) the number of identified proteins, indicating that the majority of the bacterial communities present in the phyllosphere were

genetically distinct from those currently available in databases. Most identified proteins in the phyllosphere proteome were assigned to the three bacterial genera *Methylobacterium*, *Sphingomonas*, and *Pseudomonas*. Large numbers of proteins involved in methanol oxidation were identified and were assigned to Methylobacterium species that can use methanol as a source of carbon and energy (Knief et al. 2012).

18.7 Environmental Transcriptomics

This technique allows monitoring of microbial gene expression profiles in natural environments by studying global transcription of genes by random sequencing of mRNA transcripts pooled from microbial communities at a particular time and place (Moran 2009) (Fig. 18.1). Metatranscriptomics is particularly suitable for measuring changes in gene expression and their regulation with respect to changing environmental conditions. The major challenge in metatranscriptomics is the fact that prokaryotic microbial mRNA transcripts are not polyA tailed, so obtaining complementary DNA (cDNA) is not easy (Rajendhran and Gunasekaran 2011). This results in coextraction of more abundant rRNA molecules in the total RNA pool, which can lead to overwhelming background sequences in a large-scale sequencing analysis. A method for selectively enriching mRNA by subtractive hybridization of rRNA has been developed and evaluated for the gene transcript analysis of marine and freshwater bacterioplankton communities, which revealed the presence of many transcripts that were linked to biogeochemical processes such as sulfur oxidation (soxA), assimilation of C1 compounds (fdh1B), and acquisition of nitrogen via polyamine degradation (aphA) (Poretsky et al. 2005).

More recently, a "double-RNA" method has been devised to analyze the total RNA pool of a community, as it is naturally rich in not only functionally but also taxonomically relevant molecules, i.e., mRNA and rRNA, respectively (Urich et al. 2008; Wemheuer et al. 2012). This offers a means to investigate both structural and biochemical activity of microbes in a single experiment. Their study combined transcriptomic profiling with massive pyrosequencing techniques to produce 193,219 rRNA tags and 21,133 mRNA tags from sandy soil samples that were poor in nutrients and neutral in pH. The rRNA tags providing data on the phylogenetic composition of soil microbial communities showed that Actinobacteria and Proteobacteria were most abundant, while Crenarchaeota were less abundant in soil samples (DeAngelis et al. 2011). The mRNA tags provided a glimpse of the in situ expression of several key metabolic enzymes such as ammonia monooxygenase (amoA and amoC) and nitrite reductase (nirk) that were involved in ammonia oxidation. In addition, microbial gene transcripts coding for the enzymes methylmalonyl-CoA mutase and 4-hydroxybutyryl-CoA dehydratase that play a role in CO_2 fixation pathways in Crenarchaeaota were detected.

18.8 Conclusions

Microbial diversity in natural environments is extensive. Methods for studying diversity vary and diversity can be studied at different levels, i.e., at global, community, and population levels. The molecular perspective gives us more than just a glimpse of the evolutionary past; it also brings a new future to the discipline of microbial ecology. Since the molecular-phylogenetic identifications are based on sequences, as opposed to metabolic properties, microbes can be identified without being cultivated. Consequently, all the sequence-based techniques of molecular biology can be applied to the study of natural microbial ecosystems. These methods characterize the microbial processes and thereby can be used to reach a better understanding of microbial diversity. In future, these techniques can be used to quantitatively analyze microbial diversity and expand our understanding of their ecological processes. All of the molecular approaches available for community structure and function analysis have advantages and limitations associated with them, and none provides complete access to the genetic and functional diversity of complex microbial communities. A combination of several techniques should be applied to interrogate the diversity, function, and ecology of microorganisms. Culture-based and culture-independent molecular techniques are neither contradictory nor excluding and should be considered as complementary. An interdisciplinary systems approach embracing several "omics" technologies to reveal the interactions between genes, proteins, and environmental factors will be needed to provide new insights into environmental microbiology. Development of multi-"omics" approaches will be a high-priority area of research in the coming years.

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References

Allen EE, Banfield JF (2005) Community genomics in microbial ecology and evolution. Nat Rev Microbiol 3:489–498

Banfield JF, Verberkmoes NC, Hettich RL, Thelen MP (2005) Proteogenomic approaches for the molecular characterization of natural microbial communities. OMICS 9:301–333

Barberan A, Bates ST, Casamayor EO, Fierer N (2012) Using network analysis to explore co-occurrence patterns in soil microbial communities. ISME J 6:343–351

Bergmann JC, Costa OYA, Gladden JM, Singer S, Heins R, D'haeseleer P, Simmons BA, Quirino BF (2014) Discovery of two novel β-glucosidases from an Amazon soil metagenomic library. FEMS Microbiol Lett 351:147–155

Cong J, Yang Y, Liu X, Lu H, Liu X, Zhou J, Li D, Yin H, Ding J, Zhang Y (2015) Analyses of soil microbial community compositions and functional genes reveal potential consequences of natural forest succession. Sci Rep 5:10007

DeAngelis KM, Wu CH, Beller HR, Brodie EL, Chakraborty R, DeSantis TZ, Fortney JL, Hazen TC, Osman SR, Singer ME, Tom LM, Andersen GL (2011) PCR amplification-independent methods for detection of microbial communities by the high-density microarray Phylochip. Appl Environ Microbiol 77:6313–6322

Delmont TO, Eren AM, Maccario L, Prestat E, Esen OC, Pelletier E, Le Paslier D, Simonet P, Vogel TM (2015) Reconstructing rare soil microbial genomes using in situ enrichments and metagenomics. Front. Microbiol 6:358. doi:10.3389/fmicb.2015.00358

Delmotte N, Knief C, Chaffron S, Innerebner G, Roschitzki B, Schlapbach R, von Mering C, Vorholt JA (2009) Community proteogenomics reveals insights into the physiology of phyllosphere bacteria. Proc Natl Acad Sci USA 106:16428–16433

Fakruddin M, Mannan KSB (2013) Methods for analyzing diversity of microbial communities in natural environments. Ceylon J Sci 42:19–33

Faust K, Lahti L, Gonze D, de Vos WM, Raes J (2015) Metagenomics meets time series analysis: unraveling microbial community dynamics. Curr Opin Microbiol 25:56–66

Henne A, Schmitz RA, Bömeke M, Gottschalk G, Daniel R (2000) Screening of environmental DNA libraries for the presence of genes conferring lipolytic activity on *Escherichia coli*. Appl Environ Microbiol 66:3113–3116

Hettich RL, Pan C, Chourey K, Giannone RJ (2013) Metaproteomics: Harnessing the power of high performance mass spectrometry to identify the suite of proteins that control metabolic activities in microbial communities. Anal Chem 85:4203–4214. doi:10.1021/ac303053e

Hugenholtz P. (2002) Exploring prokaryotic diversity in the genomic era. Genome Biol 3:0003

Johnson-Rollings AS, Wright H, Masciandaro G, Macci C, Doni S, Calvo-Bado LA, Slade SE, Plou CV, Wellington EMH (2014) Exploring the functional soil-microbe interface and exo-enzymes through soil metaexoproteomics. ISME J 8:2148–2150. doi:10.1038/ismej.2014.130

Knief C, Delmotte N, Chaffron S, Stark M, Innerebner G, Wassmann R, von Mering C, Vorholt JA (2012) Metaproteogenomic analysis of microbial communities in the phyllosphere and rhizosphere of rice. ISME J 6:1378–1390. doi:10.1038/ismej.2011.192

Koonin EV, Makarova KS, Aravind L (2001) Horizontal gene transfer in prokaryotes: quantification and classification. Annu Rev Microbiol 55:709–742

Kumar V (2011) Laboratory manual of microbiology. Scientific, Jodhpur, pp. 101–103

Kumar M, Varma A, Kumar V (2016) Ecogenomics based microbial enzyme for biofuel industry. Sci Int 4:1–11

Moran MA (2009) Metatranscriptomics: eavesdropping on complex microbial communities. Microbe 4:329–335

Myrold DD, Zeglin LH, Jansson JK (2013) The potential of metagenomic approaches for understanding soil microbial processes. Soil Sci Soc Am J 78:3–10

Paisie TK, Miller TE, Mason OU (2014) Effects of a ciliate protozoa predator on microbial communities in pitcher plant (*Sarracenia purpurea*) leaves. PLoS One 9:e113384. doi:10.1371/journal.pone.0113384

Pearson, WR (2013) An introduction to sequence similarity ("Homology") searching. Curr Protoc Bioinf Jun 03. doi:10.1002/0471250953.bi0301s42

Poretsky RS, Bano N, Buchan A, LeCleir G, Kleikemper J, Pickering M, Pate WM, Moran MA, Hollibaugh JT (2005) Analysis of microbial gene transcripts in environmental samples. Appl Environ Microbiol 71:4121–4126

Rajendhran J, Gunasekaran P (2011) Microbial phylogeny and diversity: small subunit ribosomal RNA sequence analysis and beyond. Microbiol Res 166:99–110

Rastogi G, Osman S, Vaishampayan PA, Andersen GL, Stetler LD, Sani RK (2010) Microbial diversity in uranium mining-impacted soils as revealed by high-density 16S microarray and clone library. Microb Ecol 59:94–108

Riesenfeld CS, Schloss PD, Handelsman J (2004) Metagenomics: genomic analysis of microbial communities. Annu Rev Genet 38:525–552

Rondon MR, August PR, Bettermann AD, Brady SF, Grossman TH, Liles MR, Loiacono KA, Lynch BA, MacNeil IA, Minor C, Tiong CL, Gilman M, Osburne MS, Clardy J, Handelsman J, Goodman RM (2000) Cloning the soil metagenome: a strategy for accessing the genetic and functional diversity of uncultured microorganisms. Appl Environ Microbiol 66:2541–2547

Rønn R, McCaig AE, Griffiths BS, Prosser JI (2002) Impact of protozoan grazing on bacterial community structure in soil microcosms. Appl Environ Microbiol 68:6094–6105

Schloss PD, Handelsman J (2004) Status of the microbial census. Microbiol Mol Biol Rev 68:686–691

Singh BK, Campbell CD, Sorenson SJ, Zhou J (2009) Soil genomics. Nat Rev Microbiol 7:756. doi:10.1038/nrmicro2119-c1

Smith CJ, Osborn AM (2009) Advantages and limitations of quantitative PCR (Q-PCR)-based approaches in microbial ecology. FEMS Microbiol Ecol 67:6–20

Streit WR, Schmitz RA (2004) Metagenomics-the key to the uncultured microbes. Curr Opin Microbiol 7:492–498

Urich T, Lanzen A, Qi J, Huson DH, Schleper C, Schuster SC (2008) Simultaneous assessment of soil microbial community structure and function through analysis of the meta-transcriptome. PLoS One 3:e2527

Vieira FCS, Nahas E (2005) Comparison of microbial numbers in soils by using various culture media and temperatures. Microbiol Res 160:197–202

Wemheuer B, Wemheuer F, Daniel R (2012) RNA-based assessment of diversity and composition of active archaeal communities in the German bight. Archaea 695826:8. doi:10.1155/2012/695826

Werner A (2014) Horizontal gene transfer among bacteria and its role in biological evolution. Life 4:217–224

Zengler K, Walcher M, Clark G, Haller I, Toledo G, Holland T, Mathur EJ, Woodnutt G, Short JM, Keller M (2005) High-throughput cultivation of microorganisms using microcapsules. Methods Enzymol 397:124–130

Zhao L, Ma T, Gao M, Gao P, Cao M, Zhu X, Li G (2012) Characterization of microbial diversity and community in water flooding oil reservoirs in China. World J Microbiol Biotechnol 28:3039–3052

Chapter 19
Arbuscular Mycorrhizal Fungi: Evolution and Functions in Alleviating Plant Drought Stress

T. Li

Abstract Arbuscular mycorrhizal (AM) fungi can form mutualistic associations known as AM symbioses with the majority of terrestrial plant species. They are essential for plant adaptation to various environmental stresses, such as nutrient deficiency, environmental pollution, and drought. Many studies have proved the positive influences of AM on plant drought tolerance, and great efforts have been made to uncover the underlying mechanisms. The progress in direct involvement of AM fungi in plant–water relations, however, is hindered by the lack of knowledge on AM fungal genetics. Here, we discuss the advantages and applicability of three tools and techniques, including comparative genomics, RNA sequencing, and noninvasive microelectrode ion flux estimation, in studying AM fungal evolutionary profiling and functioning and expect to propose new perspectives for future research.

19.1 Introduction

Arbuscular mycorrhizal (AM) symbiosis, a symbiotic association established between AM fungi and roots of higher plants, can be found in most terrestrial ecosystems (Compant et al. 2010). The fungi, as true helper microorganisms, confer many benefits to plants, such as the extension of the root system and improvement of soil nutrient uptake, especially immobile phosphates (Smith and Read 2008). Furthermore, they also can lead to a 20 % net increase in photosynthesis (Tisserant et al. 2013). In return, the fungal partner receives up to 20 % of plant-fixed carbon to support its growth (Bonfante and Genre 2010). From these perspectives, AM fungi could make a tremendous contribution to the phosphorus and carbon cycling in

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natural ecosystems. Despite the ecological importance of the AM symbiosis, its molecular and genetic features are only partially demonstrated and most in-depth investigations have centered on the plant partner (Geurts and Vleeshouwers 2012). In contrast, very little is known about the basic biology of the fungal partner due to its genetic complexity resulting from asexual reproduction and the coexistence of genetically different nuclei (Sanders and Croll 2010). The recently published genome sequence of an AM fungus *Rhizophagus irregularis* has accelerated the progress of AM fungal cellular programs. Low level of genome polymorphism was observed in the multinucleated *R. irregularis*, and some hallmarks, such as the lack of genes encoding plant cell wall-degrading enzymes and of genes involved in toxin and thiamine synthesis, have made AM fungi obligate symbionts (Tisserant et al. 2013). However, whether there are other features marking the obligate biotrophy needs to be further investigated.

Many studies have shown that the mutualistic association can protect host plants against adverse environmental conditions including drought stress, which exerts detrimental effects on plant survival, development, and productivity (Ruiz-Lozano 2003; Compant et al. 2010). It has been reasoned that AM fungi can alter water flow into and out of the plants through hyphal networks (Augé 2001; Li et al. 2013, 2014), improve nutrient uptake and photosynthesis (Ruíz-Sánchez et al. 2011), and promote plant metabolic activities, for instance, the synthesis of abscisic acid and proline (Herrera-Medina et al. 2007). Molecular evidence has demonstrated that the abovementioned mycorrhizal functions are possibly achieved through the regulation of drought-related gene expression in host plants by AM fungi, including aquaporin genes (Porcel et al. 2006), *p5cs* genes which encode a rate-limiting enzyme in the biosynthesis of proline (Porcel et al. 2004), and *nced* genes coding for a key enzyme in ABA biosynthesis (Aroca et al. 2008). Moreover, extraradical mycelium of AM fungi can enter finer pores of the soil and enhance water transport to host plants to alleviate water deficit (Khalvati et al. 2005). However, the molecular basis for the direct involvement of AM fungi in plant drought tolerance is largely unknown.

The development of many high-throughput and electrophysiological techniques that have been successfully applied to the functional and evolutionary analyses in many organisms has made it possible to answer the important biological questions regarding AM fungi. In this chapter, we discuss the applicabilities of some tools and techniques, such as comparative genomics, RNA sequencing, and noninvasive microelectrode ion flux estimation (MIFE), in AM fungi studies and expect to lay a solid foundation for future research.

19.2 Comparative Genomics: An Approach for Further Understanding the Evolution of Arbuscular Mycorrhizal Fungi

The accelerating output from the genome sequencing projects provides a wealth of data for uncovering the fundamental biological processes (Nieduszynski and Liti 2011). Each cellular function requires the precise coordination of molecular interaction networks. The assessment for the functionality of the molecular networks relies on high-quality genome annotations (Cornish and Markowetz 2014), which can be created directly by experiments or through homology-based inference (Haft 2015). In view of functional divergence within homology families (Tian and Skolnick 2003) and functional overlapping among different protein families (Koski and Golding 2001), both missed and wrong annotations exist in public databases. The best approach to high-quality genome annotation is the additional use of comparative genomics. Multiple sequence alignments in comparative genomics are superior to individual pairwise alignments for easier and more accurate identification of motif properties and thus molecular function (Schaffer et al. 2001). In addition, after initial assignment of protein family functions is performed using RAST subsystems (Aziz et al. 2008) or Genome Properties (Haft et al. 2013) and interactions among protein families are deduced in the STRING database server (Franceschini et al. 2013), comparative genomics can group proteins according to their functions at the whole genome level, even if each single protein is poorly understood. This has the potential to uncover the nature of biological processes and drive new discoveries (Haft 2015).

Comparative genomics was first used to clarify the genetic bases of mycorrhizal lifestyle evolution (Kohler et al. 2015) and suggested the substantial losses of plant cell wall degradation enzymes during mycorrhizal fungal evolution, along with the data showing genomic contents and organization of *R. irregularis* (Tisserant et al. 2013). However, the evidence of other evolutionary and functional properties is still lacking in mycorrhizal fungi.

We have run Artemis Comparison Tool v8 (Carver et al. 2005) and MUMmer 3.0 (Kurtz et al. 2004) on Linux System to compare genomes of four representative fungi with different strategies for accessing host nutrients, including the biotrophic AM fungus *R. irregularis* DAOM 197198, the necrotrophic pathogen *Fusarium verticillioides*, the ectomycorrhizal fungus *Laccaria bicolor*, and the hemibiotrophic pathogen *Magnaportheoryzae*. Our preliminary results showed that a set of genes encoding the key enzymes involved in triacylglycerol (TAG) biosynthesis were conserved in the fungi. The enzymatic steps began with the formation of glycerol-3-phosphate, which was catalyzed by glycerol kinase that transferred a phosphate from ATP to glycerol and ended with conversion of diacylglycerol to TAG catalyzed by both diacylglycerol *O*-acyltransferase and phospholipid:diacylglycerolacyl transferase. However, direct synthesis of glycerol and TAG degradation occurred only in the pathogens as the key enzymes glycerol-3-phosphatase and intracellular triacylglycerol lipase, which performed the

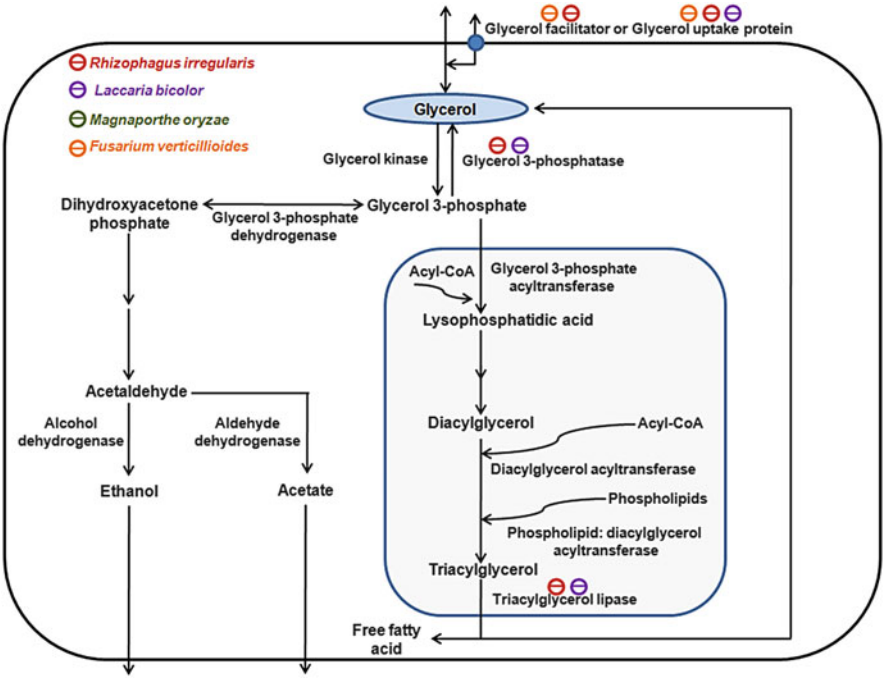

Fig. 19.1 Pathways of glycerol flow and metabolism in *Rhizophagus irregularis*, *Laccaria bicolor*, *Magnaporthe oryzae*, and *Fusarium verticillioides*. The *red*, *purple*, *olive*, and *orange* "Θ" represents the absence of the catalytic enzymes or transporters in *R. irregularis*, *L. bicolor*, *M. oryzae*, and *F. verticillioides*, respectively

catalytic conversion of glycerol-3-phosphate to glycerol and TAG to glycerol, respectively, are exclusively present in the pathogens but absent from symbiotic fungi (Fig. 19.1, unpublished data). In addition, combined with gene functional verification, we showed that the two obligatory fungi (*R. irregularis* and *F. verticillioides*) which exhibit only one type of lifestyle lacked the capacity for glycerol transport. Conversely, the two facultative fungi (*L. bicolor* and *M. oryzae*) could potentially synthesize TAG from glycerol, complete their life cycle, and thus exhibit an alternative lifestyle (Fig. 19.1). These data implied that glycerol blocks and glycerol metabolism may play crucial roles in AM symbiosis. Therefore, comparative genomics could open up the possibility of identifying new features contributing to AM symbiosis.

19.3 RNA Sequencing: A Powerful Tool to Profile AM Fungal Functioning on a Genomic Scale

At the beginning of the 2000s, the next-generation DNA sequencing approaches (Roche/454, SOLiD, and Illumina) came into use and were rapidly applied to RNA studies (Graveley 2008; Loman et al. 2012), although saddled with higher costs and less conventional protocols than other high-throughput technologies like microarrays (Ares 2011). More recently, improvements have been made to data throughput quality and expense reduction, enabling the prompt and wide utilization of RNA sequencing (RNA-Seq) in multiple experimental conditions (Marguerat et al. 2008; Wang et al. 2009). Assembly and evaluation of transcript (isoform) from the obtained sequencing reads allow discovery of novel transcripts and isoforms, detection of alternative splicing patterns and polyadenylation sites, as well as quantification of gene set expression (Marguerat et al. 2008; Wang et al. 2009; Ares 2011), providing a solid basis for transcriptome dissection. Compared with microarray analysis, RNA-seq has increased resolution and sensitivity to subtle features and differences such as transcripts at low abundance, allelic variations, and splice isoforms (Zhao et al. 2014). Furthermore, it is noteworthy that the sequencing reads from the transcriptome analysis must be mapped to their genomic origins, consequently supporting gene annotation and comparative genome analysis (Sudheesh et al. 2015).

The number of reads matching a particular gene in the genome gives an estimate of the relative expression level of that gene, which controls the phenotypic traits of living organisms (Ares 2011). Therefore, genome-wide transcriptome analysis can provide insights into the molecular basis of mycorrhizal fungal adaptation processes for growth (Tisserant et al. 2012). In fact, considerable studies have focused on the symbiosis-related mechanisms (Martin et al. 2008; Tisserant et al. 2012; Kohler et al. 2015). However, little is known about the development and functioning of AM fungi in response to abiotic stresses such as drought.

We have analyzed the transcriptome of *R. irregularis* DAOM 197198 (Tisserant et al. 2012) and found only two predicted aquaporin genes (*GintAQPF1* and *GintAQPF2*). The analysis of gene expression during different developmental stages of the life cycle of this fungus showed that the lowest expression of *GintAQPF1* and *GintAQPF2* was found in germinated spores, whereas the highest expression was identified in arbuscule-enriched cortical cells. Intermediate expression level was observed in extraradical mycelia (Fig. 19.2; Li et al. 2013). This indicates that the two transporters likely account for water uptake and water exchange between host plants and AM fungi. To determine whether there is direct involvement of AM fungi in plant–water relations under drought stress, we verified the functions and activities of the two aquaporins in heterologous expression system and evaluated their expression levels in colonized maize roots, cortical cells containing arbuscules and extraradical mycelia. Our data strongly support potential water transport via AM fungi to host plants, which leads to a better understanding of the important role of AM fungi in plant drought tolerance (Li et al. 2013). However,

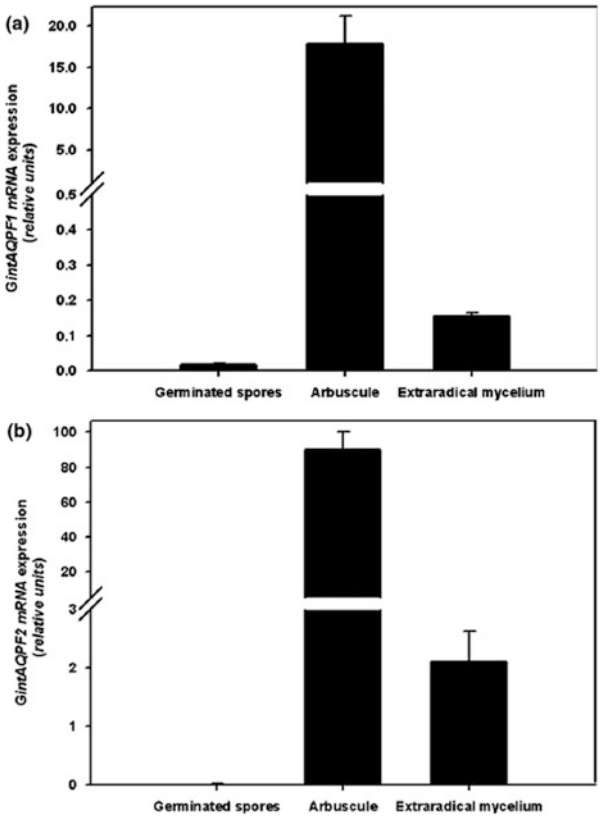

Fig. 19.2 Expression of aquaporin genes, *GintAQPF1* (**a**) and *GintAQPF2* (**b**), in germinated spores, cortical cells containing arbuscules and extraradical mycelium. The error bars represent SD

there is still an urgent need for RNA-Seq profiling under water deficit in order to provide an overview of drought-related genes of AM fungi and their regulatory networks which perceive and translate drought signals into key biological processes for drought responses.

19.4 The Application of Noninvasive Micro-test Technique for Kinetic Studies of Membrane-Transport Processes Across Cellular Membranes

Cell membranes act as semipermeable barriers that facilitate the transport of certain molecules and prevent the accumulation of undesirable chemicals (Maloney and Wilson 1996). Membrane transport is considered to be involved in the regulation of cell growth and morphology, metabolism, stress signaling, and immune response (McLamore and Porterfield 2011). Electrophysiology (Martinac 2004; Ohmizo et al. 2004) and molecular genetics (Epstein 2003; Kuo et al. 2003) studies have

revealed the critical role of plasma membrane transporters in extracellular signal perception and transduction. Plasma membrane potential alteration and/or ion flux modulations are among the earliest cellular responses to environmental changes in many organisms (Wood 1999; Zimmermann et al. 1999). Sensitive and robust technologies are needed to determine real-time quantification of cell membrane transport, based on which links between membrane-transport processes and relevant metabolic processes can be established within a specified time (Shabala et al. 2006). A variety of different techniques can be adopted to measure ion transport, consisting of nuclear magnetic resonance (NMR) spectroscopy (Gillies 1994), fluorescent indicator dyes (Jones et al. 2000; Novo et al. 2000), patch clamp (Martinac 2004), and various types of ion-selective electrodes (Orlov et al. 2002; Ohmizo et al. 2004). Challenges associated with fluorescent dyes include cytoxicity, accumulation of the fluorescence probes inside cells, and interaction of ion probes with cell metabolism (Roos 2000). The patch clamp method greatly depends on the experience and manual skills to avoid physical damage to cell membranes (Levina et al. 1999; Martinac 2004). NMR suffers from a relatively low time resolution and the restraints on types of atoms being detected (Gillies 1994).

In recent years, due to its high sensitivity and analyte selectivity, lower response time, and the novel microfabrication property, noninvasive microelectrode ion flux measurements have become a powerful tool and have advanced the progress in studying the responses of animal (Devlin and Smith 1996; Smith et al. 1999), plant (Babourina et al. 2002; Shabala and Lew 2002; Demidchik et al. 2003), yeast (Macpherson et al. 2005; Li et al. 2013), and fungal (Lew 1999; Ramos et al. 2008) cells and tissues to abiotic and biotic stresses. This newly developed system is derived from L. Jaffe's vibrating probe technique (Jaffe and Nuccitelli 1974) and could measure net fluxes of many molecules in real time, including H^+, Ca^{2+}, K^+, Na^+, Cl^-, Mg^{2+}, NH_4^+, NO_3^-, Cd^{2+}, Zn^{2+}, Cu^{2+}, O_2, H_2O_2, and auxin (IAA) (McLamore and Porterfield 2011). The theory of noninvasive MIFE technique is briefly described as follows (Newman 2001). An electrochemical potential gradient, the combined gradient of ion concentration and electrical charge, arises in the proximity of cell surface when an ion is taken up or extruded across the plasma membrane. The net ion flux can be calculated from the electrochemical potential gradient since ions in solution can move down their electrochemical potentials (see Fig. 19.3 for details, Li and Chen 2013).

Despite the popularity of the noninvasive MIFE technique in many organisms, its use in AM fungi is still very limited. Ramos et al. (2008) firstly revealed the correlation of hyphal H^+ flux with germ tube branching and lateral hyphal formation in AM fungi. More recently, we have unraveled the possible connection between Ca^{2+} flux along AM fungal lateral hyphae and their polarized growth under drought stress (unpublished data). Cells contain a series of innate mechanisms of sensing extracellular and/or intracellular molecules and modulating their transport accordingly (McLamore and Porterfield 2011). Future studies should combine the real-time transport studies with genomic and proteomic analyses, allowing us to better understand the function of AM fungi against drought stress.

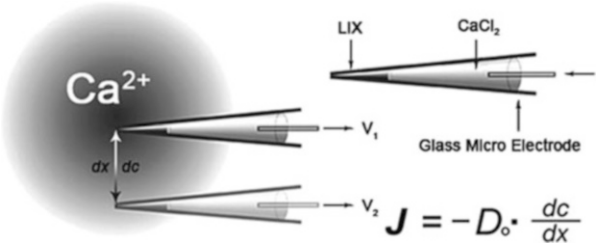

Fig. 19.3 Schematic diagram of ion flux detection (www.xuyue.net). The microelectrode tip is filled with liquid ion exchanger (LIX). A voltage gradient (*dV*) is measured by the electrometer between two positions over the travel range *dx*. A concentration gradient (*dc*) is calculated based on *dV*. D_o, ion diffusion constant; *J*, net ion flux

19.5 Conclusion

To sum up, the high-throughput techniques (comparative genomics and RNA-Seq) alone or in combination with each other can provide useful information for the evolutionary and functional studies of non-model organisms, in particular of AM fungi whose molecular genetics is still difficult to elucidate to date (Sanders and Croll 2010). Additionally, the data from noninvasive ion flux measurements should be complemented by comprehensive studies into the functional genomics of the organisms. The power of these techniques, however, might be many-fold higher when combined with other cellular or molecular techniques such as yeast two hybrid. Ultimately, we propose ways of better integrating the researches of both host plants and AM fungi in the future to unravel the functionality of this important symbiosis.

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References

Ares Jr M (2011) Methods for processing high-throughput RNA sequencing data. Cold Spring Harb Protoc. doi:10.1101/pdb.top083352

Aroca R, Vernieri P, Ruiz-Lozano JM (2008) Mycorrhizal and non-mycorrhizal *Lactuca sativa* plants exhibit contrasting responses to exogenous ABA during drought stress and recovery. J Exp Biol 59:2029–2041

Augé RM (2001) Water relations, drought and vesicular–arbuscular mycorrhizal symbiosis. Mycorrhiza 11:3–42

Aziz RK, Bartels D, Best AA et al (2008) The RAST server: rapid annotations using subsystems technology. BMC Genomics 9:75. doi:10.1186/1471-2164-9-75

Babourina O, Newman I, Shabala S (2002) Blue light-induced kinetics of H^+ and Ca^{2+} fluxes in etiolated wild-type and phototropin-mutant *Arabidopsis* seedlings. Proc Natl Acad Sci USA 99:2433–2438

Bonfante P, Genre A (2010) Mechanisms underlying beneficial plant-fungus interactions in mycorrhizal symbiosis. Nat Commun 1:48. doi:10.1038/ncomms1046

Carver TJ, Rutherford KM, Berriman M, Rajandream MA, Barrell BG, Parkhill J (2005) ACT: the Artemis comparison tool. Bioinformatics 21:3422–3423

Compant S, van der Heijden MGA, Sessitsch A (2010) Climate change effects on beneficial plant–microorganism interactions. FEMS Microbiol Ecol 73:197–214

Cornish AJ, Markowetz F (2014) SANTA: quantifying the functional content of molecular networks. PLoS Comput Biol 10:e1003808

Demidchik V, Shabala SN, Coutts KB, Tester MA, Davies JM (2003) Free oxygen radicals regulate plasma membrane Ca^{2+} and K^+-permeable channels in plant root cells. J Cell Sci 116:81–88

Devlin CL, Smith PJ (1996) A non-invasive vibrating calcium-selective electrode measures acetylcholine-induced calcium flux across the sarcolemma of a smooth muscle. J Comp Physiol 166:270–277

Epstein W (2003) The roles and regulation of potassium in bacteria. Prog Nucleic Res Mol Biol 75:293–320

Franceschini A, Szklarczyk D, Frankild S et al (2013) STRING v9.1: protein-protein interaction networks, with increased coverage and integration. Nucleic Acids Res 41:808–815

Geurts R, Vleeshouwers VGAA (2012) Mycorrhizal symbiosis: ancient signaling mechanisms co-opted. Curr Biol 22:997–999

Gillies RJ (1994) NMR spectroscopy in biomedicine. Academic, New York

Graveley BR (2008) Molecular biology: power sequencing. Nature 453:1197–1198

Haft DH (2015) Using comparative genomics to drive new discoveries in microbiology. Curr Opin Microbiol 23:189–196

Haft DH, Selengut JD, Richter RA et al (2013) TIGRFAMs and genome properties in 2013. Nucleic Acids Res 41:387–395

Herrera-Medina MJ, Steinkellner S, Vierheilig H, Ocampo Bote JA, García Garrido JM (2007) Abscisic acid determines arbuscule development and functionality in tomato arbuscular mycorrhiza. New Phytol 175:554–564

Jaffe LF, Nuccitelli R (1974) An ultrasensitive vibrating probe for measuring steady extracellular currents. J Cell Biol 63:614–628

Jones SE, Naik RR, Stone MO (2000) Use of small fluorescent molecules to monitor channel activity. Biochem Biophys Res Commun 279:208–212

Khalvati MA, Hu Y, Mozafar A, Schnidhalter U (2005) Quantification of water uptake by arbuscular mycorrhizal hyphae and its significance for leaf growth, water relations, and gas exchange of barley subjected to drought stress. Plant Biol 7:706–712

Kohler A, Kuo A, Nagy LG et al (2015) Convergent losses of decay mechanisms and rapid turnover of symbiosis genes in mycorrhizal mutualists. Nat Genet 47:410–415

Koski LB, Golding GB (2001) The closest BLAST hit is often not the nearest neighbor. J Mol Evol 52:540–542

Kuo MMC, Saimi Y, Kung C (2003) Gain-of-function mutations indicate that *Escherichia coli* Kch forms a functional conduit *in vivo*. EMBO J 22:4049–4058

Kurtz S, Phillippy A, Delcher AL, Smoot M, Shumway M, Antonescu C, Salzberg SL (2004) Versatile and open software for comparing large genomes. Genome Biol 5:R12

Levina N, Totemeyer S, Stokes NR, Louis P, Jones MA, Booth IR (1999) Protection of *Escherichia coli* cells against extreme turgor by activation of MscS and MscL mechanosensitive channels: identification of genes required for MscS activity. EMBO J 18:1730–1737

Lew RR (1999) Comparative analysis of Ca^{2+} and H^+ flux magnitude and location along growing hyphae of *Saprolegnia ferax* and *Neurospora crassa*. Eur J Cell Biol 78:892–902

Li T, Chen BD (2013) Measurement of extracellular Ca^{2+} influx and intracellular H^+ efflux in response to glycerol and PEG6000 treatments. Bio-protocol. http://www.bio-protocol.org/911

Li T, Hu YJ, Hao ZP, Li H, Wang YS, Chen BD (2013) First cloning and characterization of two functional aquaporin genes from an arbuscular mycorrhizal fungus *Glomus intraradices*. New Phytol 197:617–630

Li T, Lin G, Zhang X, Chen YL, Zhang SB, Chen BD (2014) Relative importance of an arbuscular mycorrhizal fungus (*Rhizophagus intraradices*) and root hairs in plant drought tolerance. Mycorrhiza 24:595–602

Loman NJ, Misra RV, Dallman TJ, Constantinidou C, Gharbia SE, Wain J, Pallen MJ (2012) Performance comparison of benchtop high-throughput sequencing platforms. Nat Biotechnol 30:434–439

Macpherson N, Shabala L, Rooney H, Jarman MG, Davies JM (2005) Plasma membrane H^+ and K^+ transporters are involved in the weak-acid preservative response of disparate food spoilage yeasts. Microbiology 51:1995–2003

Maloney PC, Wilson TH (1996) Ion-coupled transport and transporters. In: Neidhardt P (ed) *Escherichia coli* and *Salmonella*. ASM, Washington, DC, pp 1130–1148

Marguerat S, Wilhelm BT, Bähler J (2008) Next-generation sequencing: applications beyond genomes. Biochem Soc Trans 36:1091–1096

Martin F, Aerts A, Ahrén D et al (2008) The genome of Laccaria bicolor provides insights into mycorrhizal symbiosis. Nature 452:88–92

Martinac B (2004) Mechanosensitive ion channels: molecules of mechanotransduction. J Cell Sci 117:2449–2460

McLamore ES, Porterfield DM (2011) Non-invasive tools for measuring metabolism and biophysical analyte transport: self-referencing physiological sensing. Chem Soc Rev 40:5308–5320

Newman IA (2001) Ion transport in roots: measurement of fluxes using ion-selective microelectrodes to characterize transporter function. Plant Cell Environ 24:1–14

Nieduszynski CA, Liti G (2011) From sequence to function: insights from natural variation in budding yeasts. BBA 1810:959–966

Novo DJ, Perlmutter NG, Hunt RH, Shapiro HM (2000) Multiparameter flow cytometric analysis of antibiotic effects on membrane potential, membrane permeability, and bacterial counts of *Staphylococcus aureus* and *Micrococcus luteus*. Antimicrob Agents Chemother 44:827–834

Ohmizo C, Yata M, Katsu T (2004) Bacterial cytoplasmic membrane permeability assay using ion-selective electrodes. J Microbiol Methods 59:173–179

Orlov DS, Nguyen T, Lehrer RI (2002) Potassium release, a useful tool for studying antimicrobial peptides. J Microbiol Methods 49:325–328

Porcel R, Azcon R, Ruiz-Lozano JM (2004) Evaluation of the role of genes encoding for $\triangle^1$-pyrroline-5-carboxylate synthetase (P5CS) during drought stress in arbuscular mycorrhizal *Glycine max* and *Lactuca sativa* plants. Physiol Mol Plant 65:211–221

Porcel R, Aroca R, Azcon R, Ruiz-Lozano JM (2006) PIP aquaporin gene expression in arbuscular mycorrhizal *Glycine max* and *Lactuca sativa* plants in relation to drought stress tolerance. Plant Mol Biol 60:389–404

Ramos AC, Façanha AR, Feijó JA (2008) Proton (H^+) flux signature for the presymbiotic development of the arbuscular mycorrhizal fungi. New Phytol 198:177–188

Roos W (2000) Ion mapping in plant cells – methods and applications in signal transduction research. Planta 210:347–370

Ruiz-Lozano JM (2003) Arbuscular mycorrhizal symbiosis and alleviation of osmotic stress. New perspectives for molecular studies. Mycorrhiza 13:309–317

Ruíz-Sánchez M, Armada E, Muñoz Y, de Salamone IEG, Aroca R, Ruíz-Lozano JM, Azcón R (2011) *Azospirillum* and arbuscular mycorrhizal colonization enhance rice growth and physiological traits under well-watered and drought conditions. J Plant Physiol 168:1031–1037

Sanders IR, Croll D (2010) Arbuscular mycorrhizal: the challenge to understand the genetics of the fungal partner. Annu Rev Genet 44:271–292

Schaffer AA, Aravind L, Madden TL et al (2001) Improving the accuracy of PSI-BLAST protein database searches with composition-based statistics and other refinements. Nucleic Acids Res 29:2994–3005

Shabala S, Lew RR (2002) Turgor regulation in osmotically stressed *Arabidopsis* epidermal root cells. Direct support for the role of inorganic ion uptake as revealed by concurrent flux and cell turgor measurements. Plant Physiol 129:290–299

Shabala L, Ross T, McMeekin T, Shabala S (2006) Non-invasive microelectrode ion flux measurements to study adaptive responses of microorganisms to the environment. FEMS Microbiol Rev 30:472–486

Smith S, Read D (2008) Mycorrhizal symbiosis, 3rd edn. Academic, New York

Smith PJ, Hammar K, Porterfield DM, Sanger RH, Trimarchi JR (1999) Self-referencing, non-invasive, ion selective electrode for single cell detection of trans-plasma membrane calcium flux. Microsc Res Tech 46:398–417

Sudheesh S, Sawbridge TI, Cogan NO, Kennedy P, Forster JW, Kaur S (2015) *De novo* assembly and characterization of the field pea transcriptome using RNA-Seq. BMC Genomics 16:611. doi:10.1186/s12864-015-1815-7

Tian W, Skolnick J (2003) How well is enzyme function conserved as a function of pairwise sequence identity? J Mol Biol 333:863–882

Tisserant E, Kohler A, Dozolme-Seddas P et al (2012) The transcriptome of the arbuscular mycorrhizal fungus Glomus intraradices (DAOM 197198) reveals functional tradeoffs in an obligate symbiont. New Phytol 193:755–769

Tisserant E, Malbreil M, Kuo A et al (2013) Genome of an arbuscular mycorrhizal fungus provides insight into the oldest plant symbiosis. Proc Natl Acad Sci USA 110:20117–20122

Wang Z, Gerstein M, Snyder M (2009) RNA-Seq: a revolutionary tool for transcriptomics. Nat Rev 10:57–63

Wood JM (1999) Osmosensing in bacteria: signals and membrane-based sensors. Microbiol Mol Biol Rev 63:230–262

Zhao S, Fung-Leung WP, Bittner A, Ngo K, Liu X (2014) Comparison of RNA-Seq and microarray in transcriptome profiling of activated T cell. PLoS One 9:1–13

Zimmermann S, Ehrhardt T, Plesch G, Muller-Rober B (1999) Ion channels in plant signaling. Cell Mol Life Sci 55:183–203

Chapter 20
Engineered Nanostructured Materials for Antimicrobial and Photocatalytic Applications

Uma, Manika Khanuja, and Ajit Varma

Abstract Nanoparticles are broad spectrum antimicrobial and photocatalytic agents. This chapter reports the synthesis of few nanoparticles (ZnO, Ag, Cu-doped ZnO) for antibacterial and photocatalytic applications. The size and surface area of nanomaterials are important parameters which makes them superior than bulk materials for their antimicrobial action. In this chapter, biosynthesized ZnO nanoparticles (diameter ranging from 10 to 25 nm), Cu-doped ZnO nanorods (length ~500 nm, diameter ~50 nm), and biosynthesized Ag nanoparticles were used for antimicrobial activity. Samples were characterized by UV–Vis spectroscopy, Scanning electron microscopy (SEM), Transmission electron microscopy (TEM), Energy dispersive X-ray spectroscopy (EDX), and X-ray diffraction (XRD). Antimicrobial activity of samples is studied by shake flask method, Disk diffusion assay, and cytotoxicity by MTT assay. The significant antimicrobial activity of nanoparticles was observed for various strains like *Escherichia coli*, *Staphylococcus aureus*, *and Streptococcus pyogenes* as a function of nanoparticle concentration. The degradation of methylene blue (MB) was used as a model organic dye for photocatalytic activity. The present study demonstrates the superior photocatalytic and antibacterial activity of few nanoparticles.

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20.1 Nanostructured Materials

"Nanostructured materials" are those having properties defined by features smaller than 100 nm. Materials with a nanometer-sized microstructure are called "Nanostructured Materials" (NsM) or—synonymously—nanophase materials, nanocrystalline materials, or supramolecular solids.

This class of materials is interesting for the reasons: (1) they include most materials, since a broad range of properties—from fracture strength to electrical conductivity—depends on nanometer-scale features. (2) They may offer new properties: The conductivity and stiffness of buck tubes and the broad range of fluorescent emission of CdSe quantum dots are examples. (3) They can mix classical and quantum behaviours. (4) They offer a bridge between classical and biological branches of materials science. (5) They suggest approaches to "materials-by-design".

Nanostructured materials with good antibacterial properties and biocompatibility/environmental safety have also been attained (Campoccia et al. 2013; Anselme et al. 2010; Armentano et al. 2010; Fortunati et al. 2013). Although the design and development of biomaterials have significantly advanced over the past decade, great challenges remain for fundamental exploration and practical applications.

20.2 Engineered Nanostructured Materials

The evolution and potentiality of emergent active engineered nanostructured materials with their antibacterial and photocatalytic applications is described in this chapter. Figure 20.1 shows a schematic view of the two approaches used to develop engineered nanostructured polymeric materials for antibacterial applications: (1) nanocomposites based on biodegradable polymers and antimicrobial nanostructures with potential application in tissue engineering and (2) surface modifications based on plasma treatment in order to induce specific surface topography that affects the microbial vitality.

20.2.1 Biosynthesized ZnO

Biosynthesized nanoparticles are gaining attention due to their low cost, simplicity, and eco-friendly nature. ZnO has broad range of applications including antimicrobial activity due its biocompatibility, enhanced surface to volume ratio, wide range of morphologies, surface defects and surface charge and tunable band gap. ZnO is considered as food safe (GRAS) by food and drug administration. Through biosynthesis, i.e., by using eco-friendly, nano-toxic, and safe reagents, controlled and

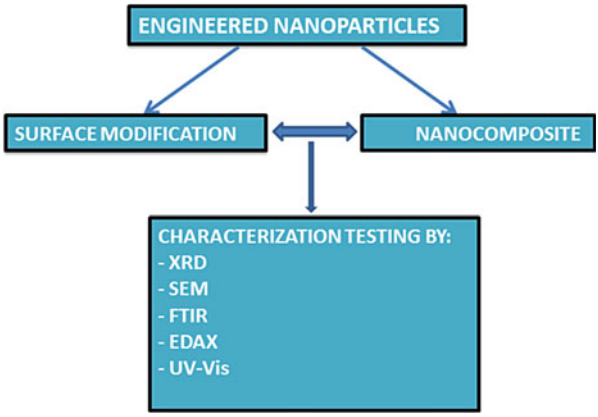

Fig. 20.1 This figure shows a schematic view of the two approaches used to develop engineered nanostructured polymeric materials for antibacterial applications

precise synthesis of metal and metal oxide nanoparticles with well-defined diverse sizes and shapes is achievable.

Green synthesis of ZnO nanoparticles were done using 25 % (w/v) of *Azadirachta indica* (Neem) leaf extract dried and was characterized further using TEM, EDX, UV–Vis, and XRD as summarized in Fig. 20.2 (Bhuyan et al. 2015a).

20.2.2 Biosynthesized Silver Nanoparticles

Silver nanoparticles (Ag NPs) exhibit unique and tunable surface plasmon properties, ease of surface functionalization, extremely high surface to volume ratio, and catalytic effect in many important oxidization reactions (Linic et al. 2015; Wiley et al. 2007). These characteristics promote their broad functions in diverse applications ranging from targeted drug delivery and molecular imaging to water treatment and antimicrobial development (Arvizo et al. 2012; Huang et al. 2008). One of the key aspects that define its suitability for these applications is the synthesis protocol. Several physical and chemical methods—including reduction of silver salt solution, thermal decomposition of silver compound, and sonication—have been reported in the literature and are currently used for producing a palette of designed nanostructures (Chen et al. 2009; Shamim and Sharma 2013; Xie et al. 2007a, b). Yet, despite the ease of such fabrication methods and their reliability in creating complex morphology of Ag NPs, toxicity and biocompatibility concerns have severely impeded their application in critical domains, e.g., in healthcare theranostics. To meet this technological need, investigators have proposed the use of microbial plateforms as a cleaner, "green," and sustainable route. Biogenic nanoparticles production reduces (and often eliminates) the need for employing hazardous chemicals and decreases the downstream processing requirements, which make the complete process more economical and less energy intensive (Raveendran et al. 2003; Prasad 2014; Prasad et al. 2015; Thakkar et al. 2010).

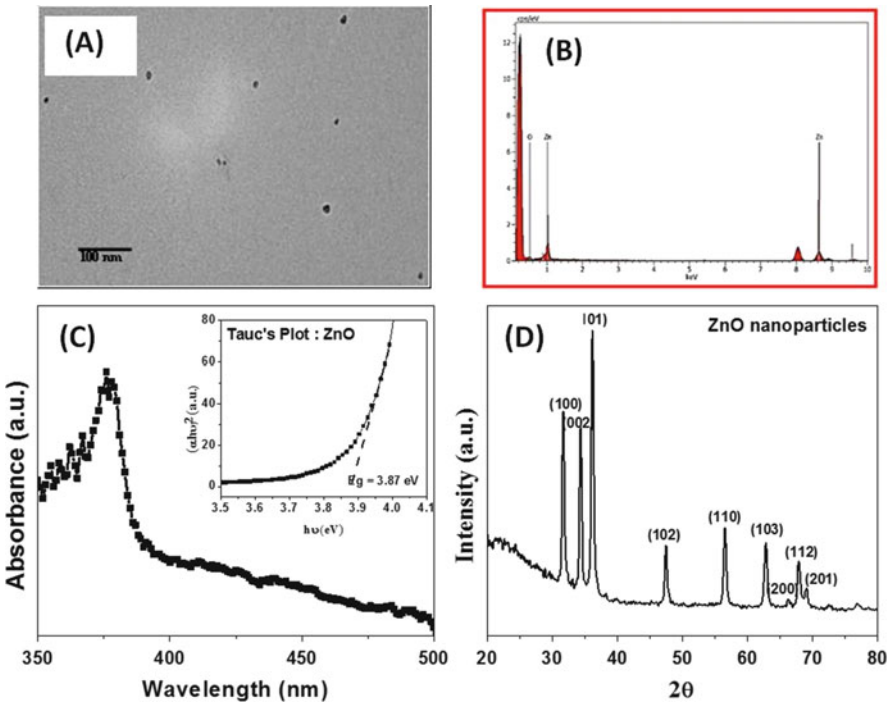

Fig. 20.2 Biosynthesized ZnO characterized by (**a**) transmission electron microscopy for particle size (10–25 nm) (**b**) EDX spectra confirming presence of elements Zn and O (**c**) UV–Vis absorption spectra and insert shows Tauc's plot, estimated band gap (E_g) = 3.87 eV and (**d**) X-ray diffractogram showing (hkl) planes corresponding to hexagonal wurtzite structure (Bhuyan et al. 2015a)

For this purpose, Ag NPs have been synthesized using *Chlorella pyrenoidosa* and was characterized by SEM, EDX, XRD, TEM, and FTIR as shown in Fig. 20.3 (Aziz et al. 2015).

20.2.3 Cu-Doped ZnO (Nanocomposite)

Since ages, copper is known for its antimicrobial action. Till date, there are no reports that bacteria's have developed antimicrobial resistance against copper. Thus, synergetic association of copper and zinc in "copper-doped ZnO nanorods" has the potential to overcome the challenge associated with AMR. This is due to (1) copper doping tunes the ZnO band gap such that effective utilization of sunlight is more for the generation of superoxide anions and (2) by changing morphology of ZnO from "spherical" in nanoparticles to 1-D nanostructures in ZnO nanorods result in increase in spatial confinement of electron and hole, thus reducing electron

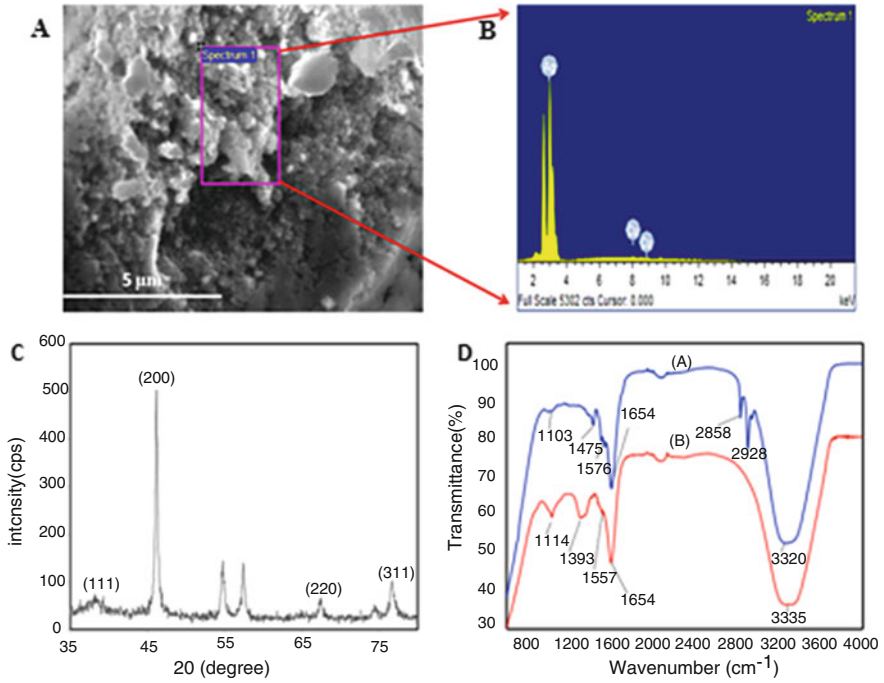

Fig. 20.3 Morphological and chemical characterization of *C. pyrenoidosa*-derived Ag NP. (**a**) SEM image of biogenic Ag NPs (scale bar indicates 5 µm); (**b**) EDX spectrum of biogenic Ag NPs; (**c**) XRD spectrum showing the face centered cubic (FCC) nature of the *C. pyrenoidosa* derived Ag NPs; (**d**) FTIR spectra of (**a**) cell extract and (**b**) *C. pyrenoidosa*-derived Ag NPs (Aziz et al. 2015)

hole recombination probability making them available for the formation of superoxide anions. Copper-Doped ZnO nanostructures have been synthesized using mechanical assisted thermal decomposition process and was characterized further using SEM, UV–Vis, and XRD as summarized in Fig. 20.4 (Bhuyan et al. 2015b).

20.3 Antimicrobial Activity

Antibacterial activity of nanoparticles is of tremendous practical applications in designing microbial resistant articles for preserving food and wood products, cosmetics, novel nanomedicines wound dressing, and disinfecting agents.

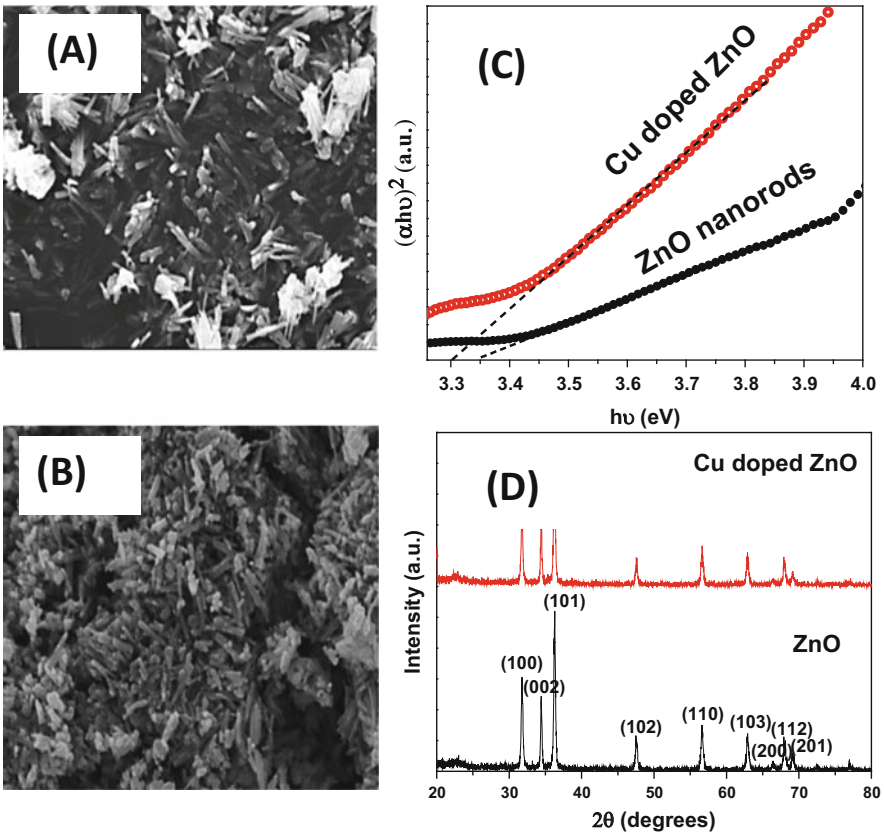

Fig. 20.4 (**a**) and (**b**) SEM micrograph of ZnO and Cu-doped ZnO nanorods, respectively; (**c**) UV–Vis of ZnO and Cu-doped ZnO; (**d**) XRD pattern of ZnO and Cu-doped ZnO nanorods (Bhuyan et al. 2015b)

20.3.1 Scanning Electron Microscopy

SEM analysis of the prepared bacterial samples was carried out showing the changes in external morphologies of the bacterial strain. Figure 20.5 shows the SEM micrograph of *E. coli* in (i) control, (ii) Cu-doped ZnO nanorods, (iii) control for Carbon nanotubes, and (iv) Carbon nanotubes. The untreated *E.coli* cells (control) seemed to retain their rod-shaped morphology with intact and well-preserved cell walls and membranes as shown in Fig. 20.5 (i and iii). In contrast, Fig. 20.5 (ii) shows the aggregated colonies of dead bacterial cells upon interaction with Cu-doped ZnO nanorod samples. Carbon nanotubes-treated *E. coli* cells undergo cell wall degradation resulting in the release of cytoplasmic contents as shown in Fig. 20.5 (iv).

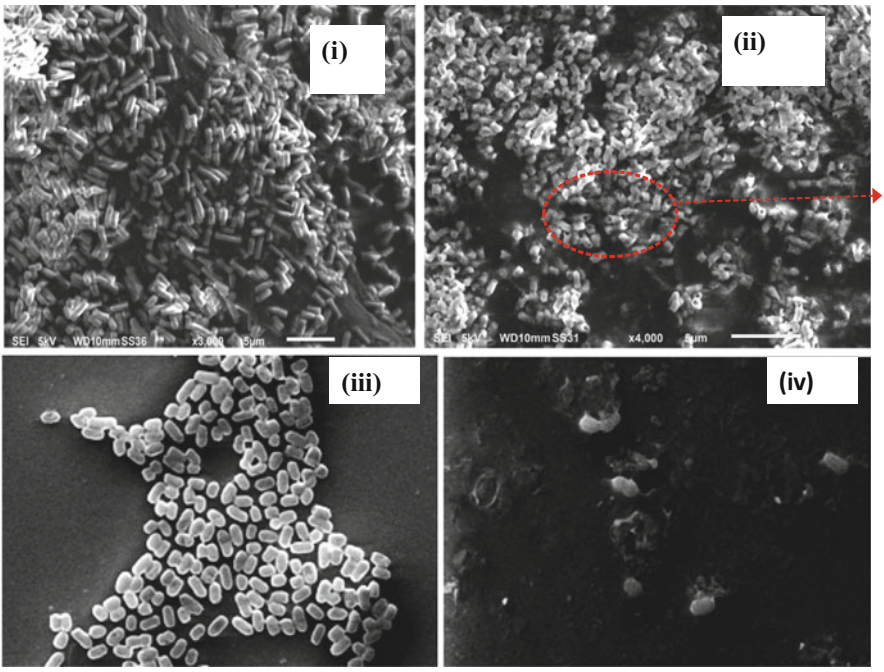

Fig. 20.5 SEM micrograph of *E. coli* in (i) control (ii) Cu-doped ZnO nanorods (iii) control and (iv) Carbon nanotubes (c.r.f. Bhuyan et al. 2015b)

20.3.2 Disk Diffusion Assay

To study the antibacterial activity of biosynthesized Ag NPs, we tested them against four pathogenic microorganisms, *Klebsiella pneumoniae, Aeromonas hydrophila*, *Acenetobacter* sp. (all of which are Gram-negative bacteria), and one Gram-positive bacterium *Staphylococcus aureus*. The chemically synthesized Ag NPs were used as controls for comparison with the activity of the biogenic materials. As seen from Fig. 20.6a, the highest zones of inhibition were observed in the cases of *A. hydrophila* and *Acenetobacter* sp. even at comparably lower volumes of added Ag NPs. The gross visual inspection is confirmed by the quantitative morphometric analysis of the inhibitory effect (Fig. 20.6b). Specifically, the percentage inhibition for *A. hydrophila* and *Acenetobacter* sp. was computed to be in the range of 15–23 % over the range of added Ag NPs volumes. On the other hand, *K. pneumonia* exhibited comparatively lower inhibition; however, it showed the sharpest rise in inhibitory effect as a function of added amount of Ag NPs.

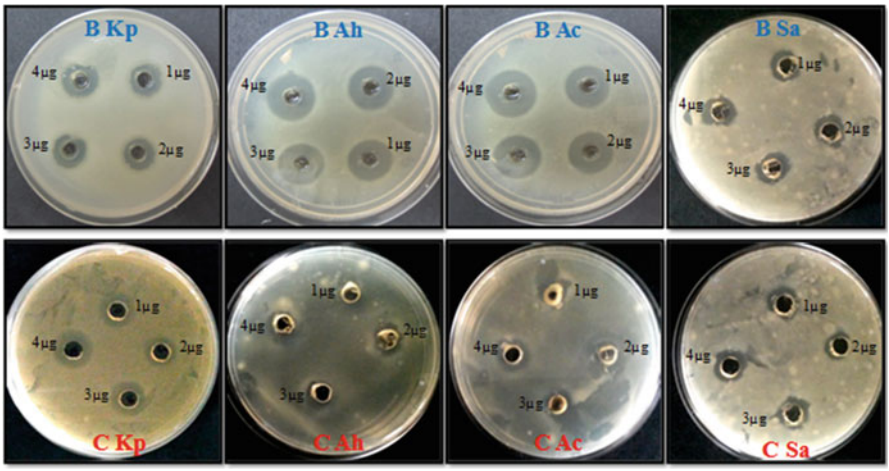

Fig. 20.6 Experimental observations of the antibacterial property of *C. pyrenoidosa*-derived Ag NP (**b**) and chemically synthesized Ag NPs (**c**). Zone of inhibition against bacterial pathogens: *Klebsiella pneumoniae* (Kp), *Aeromonas hydrophila* (Ah), Acenetobacter sp. (Ac), and *Staphylococcus aureus* (Sa) (Aziz et al. 2015)

20.3.3 Shake Flask Test in Luria Bertani (LB) broth

Preparation of Bacterial Cultures

The antibacterial assays of the nanorod samples were determined against both Gram-positive bacteria: *Staphylococcus aureus* and *Streptococcus pyogenes*, and Gram-negative bacteria: *Escherichia coli*. The stock bacterial cultures were maintained at 37 °C. Sterile Luria Bertani (LB) broth of 100 ml was prepared in 500 ml Erlenmeyer flasks followed by inoculation of single bacterial colonies from each of the bacterial stock cultures of *Staphylococcus aureus*, *Streptococcus pyogenes*, *and Escherichia coli*. Finally, the prepared bacterial suspensions were placed in an incubator shaker at 37 °C.

Shake Flask Method

Shake flask test in Luria Bertani (LB) broth.

20.3.3.1 Biosynthesized ZnO

The antibacterial activity of biosynthesized ZnO nanoparticles is tested on gram-negative as well as gram-positive bacteria: *S. aureus, S. pyogenes and E. coli*. The studies have been carried out for different concentrations (µg/ml) of ZnO (0, 20, 40, 60, 80, 100), as summarized in Table 20.1. The growth of bacterial cells is inhibited in the presence of ZnO as compared to ZnO. The inhibition in growth is observed to be 70 % for *E. coli*, 57 % for *S. aureus*, and 52 % for *S. pyogenes* as

Table 20.1 Antibacterial activity of ZnO nanoparticles (using nutrient broth shake flask test) (Bhuyan et al. 2015a)

Concentration of ZnO nanoparticles (μg/ml)	Test organisms Optical density (OD) after 24 h (600 nm)					
	E. coli (OD)	Growth reduction (%)	*S. aureus* (OD)	Growth reduction (%)	*S. pyogenes* (OD)	Growth reduction (%)
0	4.6	100.0	5.2	100.0	2.2	100.0
20	4.3	92.4	5.1	97.0	2.1	94.0
40	3.9	83.7	3.4	76.0	1.7	77.4
60	3.7	79.6	3.7	70.4	1.2	56.6
80	3.6	77.7	3.1	60.4	1.1	51.6
100	3.2	69.2	3.0	57.0	1.1	52.0

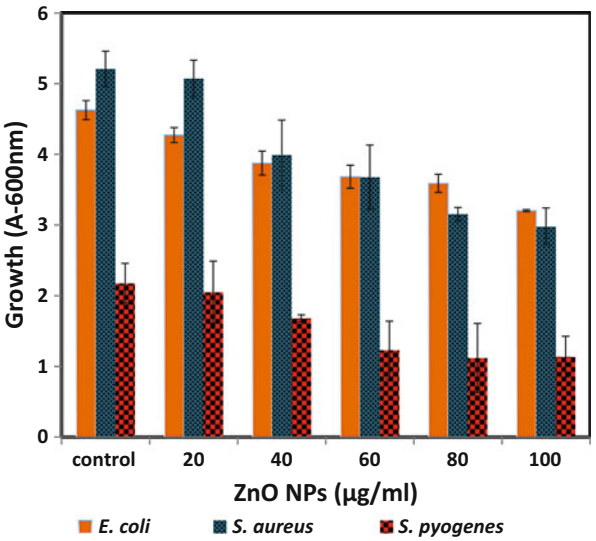

Fig. 20.7 Growth of bacterial strains (*S. aureus*, *S. pyogenes*, and *E. coli*) exposed to various concentrations of ZnO nanoparticles (20–100 μg/ml). Values plotted are mean ± Standard deviation (Bhuyan et al. 2015a)

shown in Fig. 20.7. The inhibition in bacterial cells' growth is more as concentration of biosynthesized ZnO is increased from 20 to 100 μg/ml.

20.3.3.2 Cu-Doped ZnO Nanorods

The antibacterial assay of the copper-doped ZnO nanorods was determined against both Gram-positive and Gram-negative bacterial species: *Staphylococcus aureus*, *Streptococcus pyogenes*, and *Escherichia coli*. Antibacterial activity of CZN sample was carried out using shake flask method as a function of time (0, 3, 6, 9, and 24 h) against Gram-Positive: *S. aureus and S. pyogenes* as well as Gram-Negative: *E. coli* (Fig. 20.8). Percentage reduction in bacterial growth was found to be: CZN

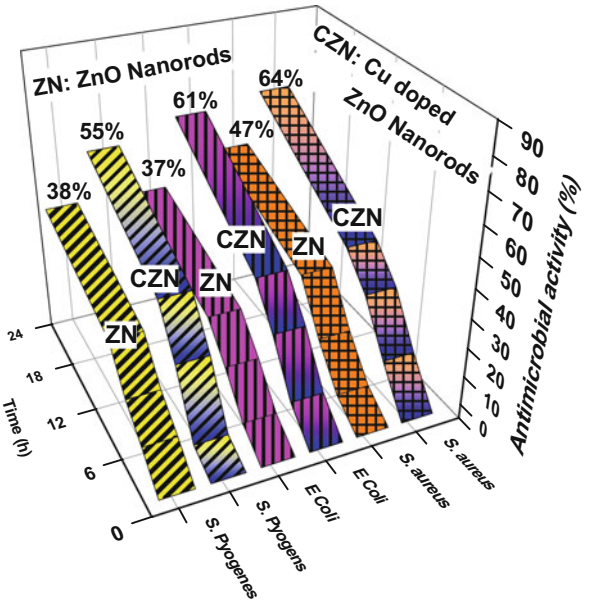

Fig. 20.8 Comparative analysis of the antibacterial efficacy of pure ZnO and Cu-doped ZnO nanorods using shake flask method (Bhuyan et al. 2015b)

Table 20.2 Antibacterial activity of pure and copper-doped ZnO nanorods (Bhuyan et al. 2015b)

	Antibacterial activity (%)					
	E. Coli		*S. Aureus*		*S. Pyogenes*	
Time (h)	Sample ZN (%)	Sample CZN (%)	Sample ZN (%)	Sample CZN (%)	Sample ZN (%)	Sample CZN (%)
0	0	0	0	0	0	0
3	8.3	6.1	6.2	3.7	8.5	13.4
6	15.5	16.9	17.9	22.5	23.7	28.9
9	27.2	26.4	32.8	36.6	35.5	38.2
24	37.7	36.9	46.6	54.7	60.8	64.2

ZN ZnO nanorods, *CZN* Copper-doped ZnO nanorods

E. coli (61 %), CZN *S. aureus* (64 %), and CZN *S. pyogenes* (55 %). The doped ZnO samples are effective antibacterial agents on Gram-Positive as well as on Gram-negative bacteria as summarized in Table 20.2. The antimicrobial action of sample was found to be highest for *S. aureus* as compared to *E. coli* and *S. pyrogenes* due to the differences in (1) cell membrane structure, (2) Physiology and metabolic activities of the cell, and (3) degree of contact.

20.3.4 MTT Assay

Cytotoxic effect of nanoparticles was determined by 3-(*4*,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide (MTT) assay. The effect of different

Table 20.3 Percentage of cell viability OF $BiVO_4$ nanoparticle against *E. coli*

Concentration of $BiVO_4$ (ppm)	Sample absorbance	Cell viability (%)	Cytotoxicity (%)
Control	0.467	100	0
20	0.133	28.47	71.53
40	0.061	13.06	86.94
60	0.057	12.2	87.8

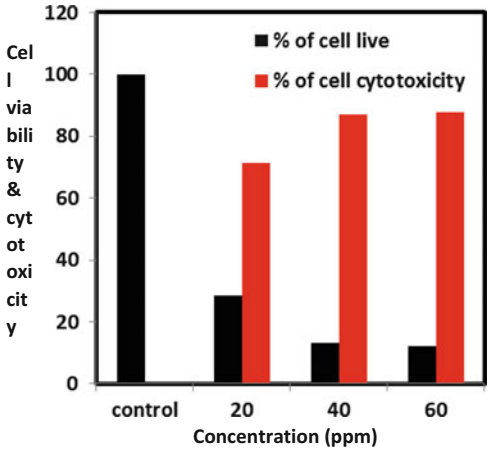

Fig. 20.9 The antibacterial activity of different concentrations of m-$BiVO_4$ nanoparticles against *E. coli* by MTT assay

concentrations of BiVO4 nanoparticles against *E. coli* was studied by MTT assay. Table 20.3 summarizes the antibacterial activity of $BiVO_4$ nanoparticle. It can be inferred from the Fig. 20.9 that the growth of bacterial cells in presence of $BiVO_4$ nanoparticles was lower than that of cells in the control, indicating that $BiVO_4$ nanoparticles could inhibit the growth of bacterial cell. The percentage of bacterial growth decreased to 71–88 % for *E. coli* with the increase in $BiVO_4$ Conc., respectively. The absorbance of nanoparticle-treated *E. coli* culture is measured at 600 nm with respect to control. Cell viability was determined by

$$\text{Cell Viability}\,(\%) = (A_t/A_c) \times 100$$
$$\text{Cytotoxicity}\,(\%) = 100 - \text{Viability}\,(\%)$$

where A_t is the absorbance of treated sample and A_c is the absorbance of control.

20.3.5 Cytotoxicity of Catechin–Cu NPs and Catechin

HepG2 cells were exposed to Catechin–Cu nanoparticles and Catechin at concentrations of 0, 1, 5, 10, 20, 40, 100, and 200 ppm for 24 h, and cytotoxicity was

Table 20.4 Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of Catechin solution and Catechin–Cu nanoparticles against various microorganisms (Huanhuan et al. 2015)

	Catechin		Catechin–Cu nanoparticles	
Bacteria	MIC (ppm)[a]	MBC (ppm)[b]	MIC (ppm)[a]	MBC (ppm)[b]
E. coli	100	150	20	40
S. aureus	90	100	10	20

[a]Concentration (three replications) where no turbidity was observed in culture
[b]Concentration (three replications) where no growth was observed on agar plate

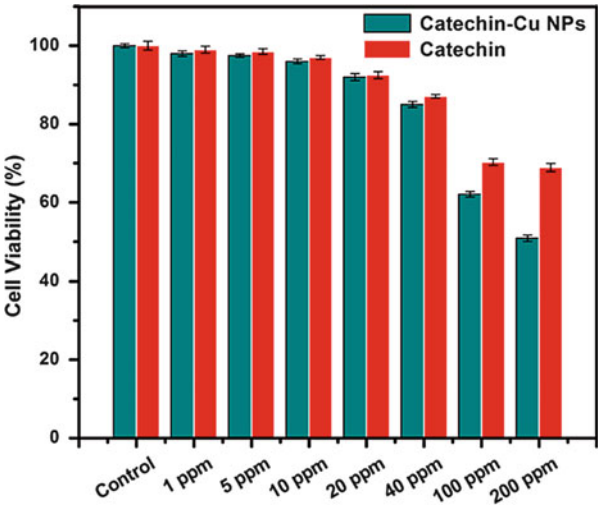

Fig. 20.10 Cytotoxicity of Catechin–Cu nanoparticle and Catechin in HepG2 cells as assessed by MTT assay (Huanhuan et al. 2015)

determined by MTT assay. The cytotoxicity assays have shown that Catechin–Cu nanoparticles up to the concentration of 40 ppm did not produce significant cytotoxicity to cells ($P > 0.05$ for each), a dose that was found to be lethal for the bacteria tested in this study (Table 20.4). As the concentration of nanoparticles increased to 100 and 200 ppm, in MTT assay, cell viability dropped drastically to 62.1 % and 59.2 %, respectively (Fig. 20.10). Furthermore, the cell viability kept about 70 % in the presence of 100 ppm Catechin, a dose that the MBC of Catechin to *S. aureus*. Catechin–Cu nanoparticles show the higher cytotoxicity to HepG2 cells than that of Catechin at the concentration range of 1–200 ppm, which can better inhibit the proliferation of HepG2 cells. Such cytotoxicity induced by Catechin–Cu nanoparticles should be attributed to the direct interaction of nanoparticle with the HepG2 cells; the result suggested that small size is a major advantage of Catechin–Cu nanopaticles (Huanhuan et al. 2015).

20.4 Antimicrobial Mechanism

The mechanism of toxicity of the Cu and ZnO nanoparticles mainly involves unfolding of proteins, loss of enzymatic activity, and thiol cross-linking and depends on nanoparticle-biomolecule interaction (Moos et al. 2010). The diffusion of nanoparticles across the cell membrane occurs due to the presence of positive ions or other variables on the surface of nanomaterials and the small size of the Cu and ZnO nanoparticles (Verma et al. 2008; Nel et al. 2009). The precise mechanism revealing the mechanism of toxicity of metal oxide nanoparticles still remains unclear. However, four mechanisms potentially explain the pathways of cellular toxicity of nanostructured Cu and ZnO after their entry into the cell: (1) oxidative stress, (2) coordination effects, (3) contact killing, and (4) non-homeostasis effects (Chang et al. 2012) as shown in Fig. 20.11.

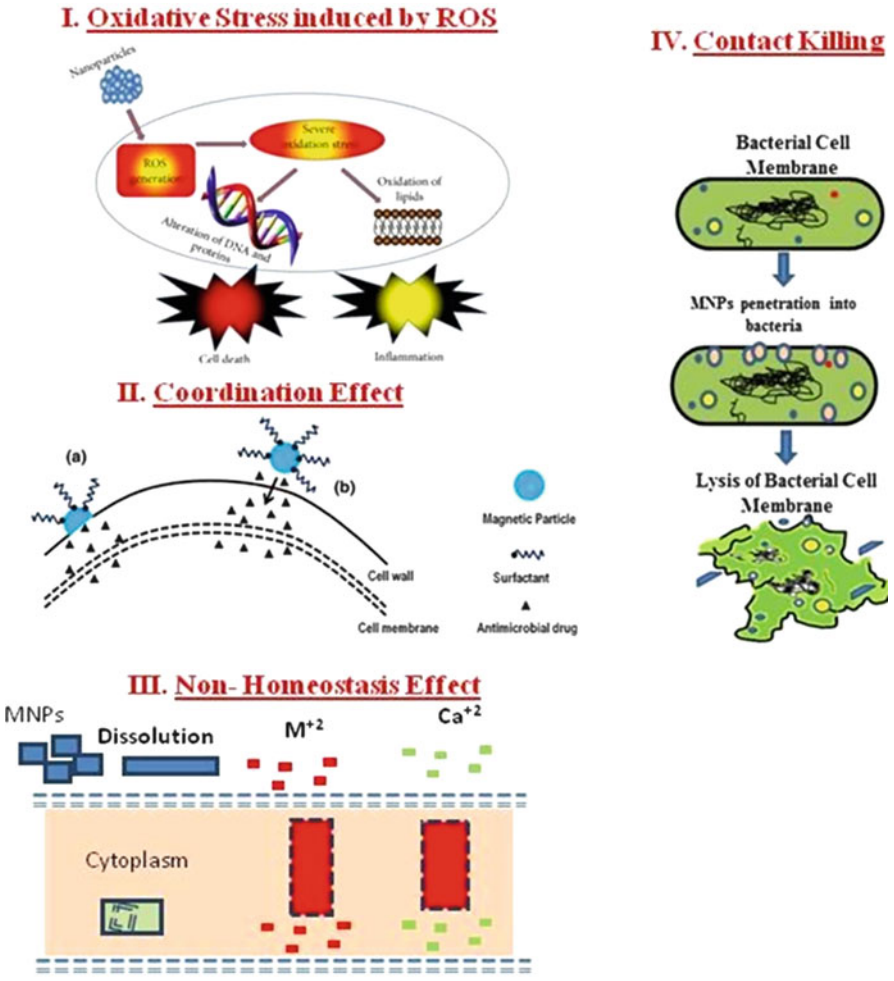

Fig. 20.11 Mechanism of antimicrobial action of Cu–ZnO nanoparticle

20.4.1 Oxidative Stress

The major toxicological mechanisms of ambient nanoparticles include generation of reactive oxygen species (ROS) and induction of intracellular oxidative stress by creating imbalance between oxidant and antioxidant processes (Chang et al. 2012). Nanoparticles induce ROS generation in eukaryotic cells; these radicals cause severe oxidation stress in the cells, affecting membrane lipids and altering the structure of DNA and proteins. This excess radical production induces an inflammatory process that could lead to cell death.

20.4.2 Coordination Effects

Coordination and non-covalent interactions occurs directly due to the interactions between metal oxide NPs and proteins in vivo or in vitro resulting in major structural changes of proteins, reduction in protein function, decreasing the α-helical content of free protein and many other protein abnormalities (Ueno et al. 2007). Moreover, several biomolecule contains coordination atoms at their active sites (mainly O and N atoms) that form chelates with Cu^{2+} and Zn^{2+} by donating lone pair of electrons. The resulting NP-biomolecule coordination inactivates the functional biomolecule by affecting the normal physiology process, promoting cellular DNA damage, and then consequently resulting in cellular toxicity (Rao et al. 2010). In addition, metal ions (Cu^{2+} and Zn^{2+}) released by NPs interacts with mRNA stabilizing proteins leading to degradation of cytoplasmic mRNA and hinder cellular transcription–translation processes (Soenen et al. 2010).

20.4.3 Contact Killing

The accumulation of the metal oxide nanoparticles in the bacterial membrane results in membrane disorganization due to the subsequent release and binding of metal ions to the membrane. However, the toxicity of nanostructured Cu and ZnO is not directly related to their entry inside the cell, rather, to a certain extent, is related to their close contact onto the cell causing changes in the vicinity of organism–particle contact area. Nanoparticles fuse with microbial cell wall or membrane; b nanoparticles bind to cell wall and release drug molecules, which will diffuse into the interior of the microorganisms.

This results in an increase of solubilization of metal and generation of ROS leading to cell death (Walch et al. 2014). Santo et al. (2011) reported that the primary targets for contact killing through surface-released copper ions are the cell membranes. The metallic ions cause the inactivation of some enzymes (e.g., hydratase in *E. coli*) necessary for normal cell function and also damage the exposed Fe–S clusters resulting in inhibition of bacterial cell growth (Macomber and Imlay 2009).

20.4.4 Non-homeostasis Effects

Metal ions play important roles in maintaining homeostasis of organisms in an independent manner and also keeping the composite functioning (Galhardi et al. 2005). However, any variation in local concentration of these metal ions disrupts metal cation cellular homeostasis ultimately resulting in occurrence of toxicity. It has been reported that the release of Cu^{2+} and Zn^{2+} ions by CuO and ZnO NPs enhances the local concentration of intracellular metal ions accelerating induction of high oxidative stress. This in turn alters the release rate of intracellular Ca^{2+}, which leads to mitochondrial perturbation, imbalance in cellular processes, and ultimately cell death (Xia et al. 2008).

20.5 Photocatalysis

Photocatalytic activity of nanoparticles offers a promising method for waste water treatment (Srinivasa et al. 2012). Methylene blue is one of the most common organic pollutants discharged from the industries directly or indirectly into water sources causing water pollution (Ameta et al. 2013). ZnO Nanoparticles exhibit very good photochemical reactivity and efficiently degrading toxic water pollutants released from textile and dying industries by utilizing natural source of energy and sunlight. This is due to the presence of many active sites and fabrication of hydroxyl radicals on ZnO surface (Baruah et al. 2009; Kajbafvala et al. 2012).

20.5.1 Cu-Doped ZnO

The photocatalytic activity was performed by employing the synthesized ZnO and Cu-doped ZnO nanorods as photocatalysts in order to study the degradation of aqueous solution of methylene blue dye. It is evident that Cu-doped ZnO nanorods leads to more efficient degradation of MB for the same exposure time as compared to pure ZnO. Figure 20.12a shows the degradation efficiency (ŋ) of both ZnO and Cu-doped ZnO nanorods as a function of time (30, 60, 90, and 120 min). The

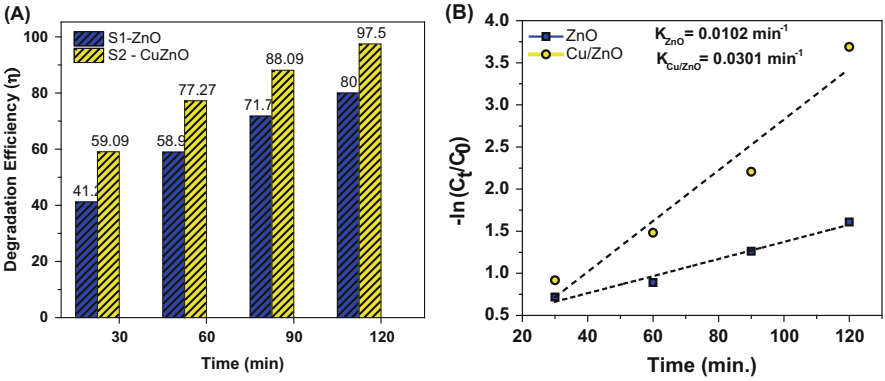

Fig. 20.12 (**a**) Degradation efficiency (η) as a function of time (30, 60, 90, 120 min) and corresponding (c.r.f. Bhuyan et al. 2015b) (**b**) Photodegradation kinetics of MB catalyzed by pure ZnO and Cu-doped ZnO nanorods (c.r.f. Bhuyan et al. 2015b)

percent degradation of MB under UV irradiations using ZnO nanorods was 41.2 %, 58.9 %, 71.8 %, and 80.0 %, respectively, at 30, 60, 90, and 120 min, respectively. Interestingly, as compared to ZnO nanorods, Cu-doped ZnO nanorods exhibited higher photocatalytic activity degrading the methylene blue upto 97.5 % of the initial value at the end of 120 min. The percent degradation of MB using Cu-doped ZnO nanorods was recorded to be 59.1 %, 77.3 %, 88.1 % and 97.5 % at 30, 60, 90, and 120 min, respectively. Study of the photo degradation kinetics of methylene blue (MB) catalyzed by pure and Cu-doped ZnO nanorods are shown in Fig. 20.12b. The photocatalytic activity follows the pseudo-first-order kinetics and is given by

$$C_t = C_0 e^{-kt}$$

Alternatively, $\ln C_0/C_t = kt$

where C_0 is the initial concentration, C_t is the concentration after time t, and k is the rate constant of pseudo-first-order reaction (Mohan et al. 2012a, b). A linear relationship between ln (C_0/C_t) and time (t) has been observed. The first-order degradation rate constants for pure ZnO and Cu-doped ZnO are found to be k ZnO = 0.0102 min^{-1} and kCu/ZnO = 0.0301min^{-1}, respectively. Thus, degradation rate of MB by Cu-doped sample is three times faster as compared to pure ZnO.

20.5.2 Biosynthesized ZnO

The photocatalytic activity was carried out by employing the biosynthesized ZnO nanoparticles as a photocatalyst in order to study the degradation of aqueous

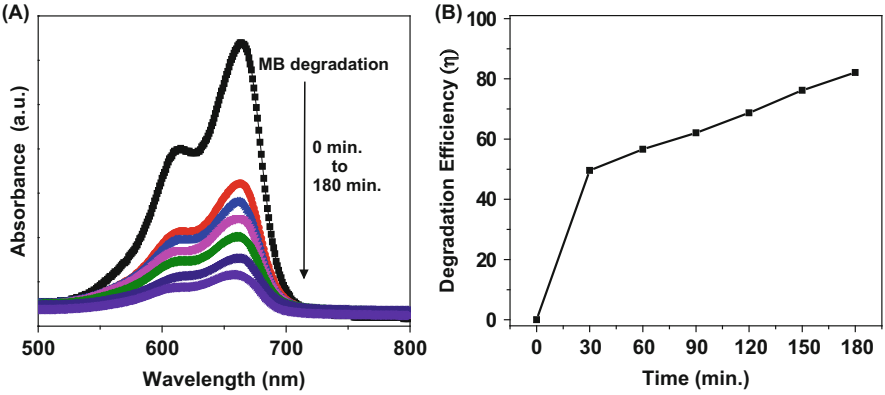

Fig. 20.13 (**a**) UV–Vis absorption spectra of methylene blue showing photocatalytic degradation at different time intervals using biosynthesized ZnO NPs as a photocatalyst. (**b**) Photocatalytic degradation efficiency of biosynthesized ZnO nanoparticles for methylene blue as a function of time

solution of methylene blue dye. The photocatalytic process involves three steps (1) generation of electron hole pairs, (2) separation of electron hole pairs, and (3) surface redox reaction on the surface for the generation of highly active hydroxyl (OH) radicals (Yu et al. 2013). These radicals act as powerful agents for oxidizing dyes like MB when placed near the surface of the photocatalyst. Figure 20.13a shows the absorption spectra of methylene blue taken at different times (0, 30, 60, 90, 120, 150, 180 min). It has been observed that the characteristic absorption peak of MB at 664 nm diminishes sharply after first 30 min, followed by the gradual decrease with time (30–180 min) on exposure to UV light. The degradation efficiency (n) of ZnO nanoparticles was plotted as a function of time shown in Fig. 20.13b. The percent degradation of MB under UV irradiations was 49.6 %, 56.6 %, 62.1 %, 68.7 %, 76.2 %, 82.1 %, at 0, 30, 60, 90, 120, 150, and 180 min, respectively. Interestingly, Methylene blue was degraded to 50 % of its initial value within first 30 min of exposure. This is in good match with the observed decolorization of MB in first 30 min. At the end of the reaction (after 180 min), MB was degraded to 82 % of its initial value. This study provides proof of concept for ZnO nanoparticles to be used as efficient photocatalyst in environmental remediation applications.

20.5.3 Silver Photocatalysis

The photocatalytic activity of biosynthesized Ag NPs was evaluated by degrading methylene blue (MB) dye under visible light irradiation. Since MB is a recalcitrant organic molecule resistant to biological degradation and its presence in water presents a serious threat to aquatic life, photocatalytic degradation of MB is both

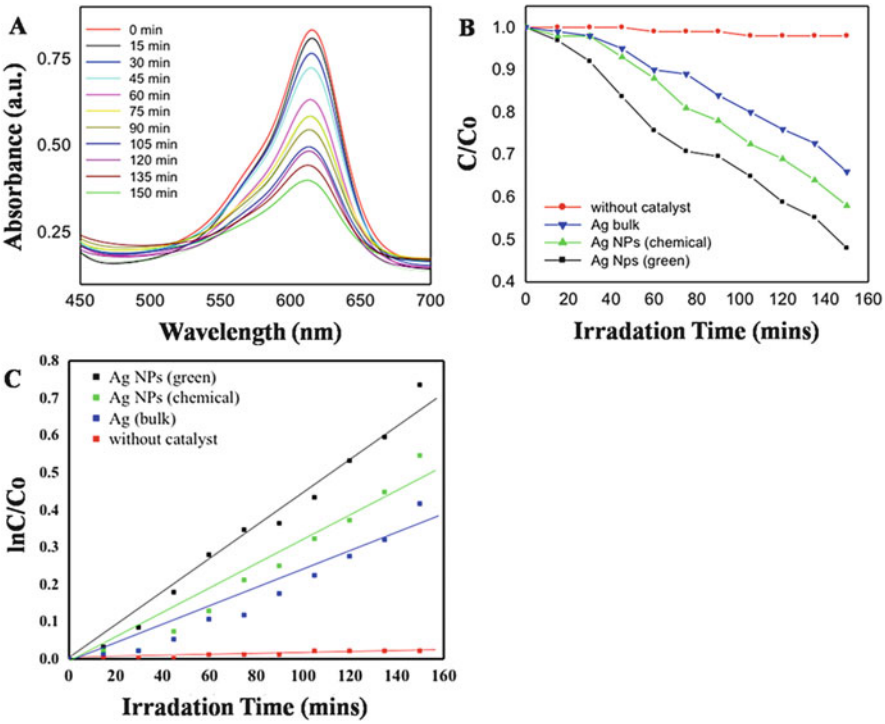

Fig. 20.14 Photocatalytic activity of *C. pyrenoidosa*-derived Ag NPs. (**a**) Degradation of methylene blue (MB) dye under visible light irradiation using biogenic Ag NPs; (**b**) Plot of MB photodegradation versus irradiation time, as measured by optical absorption; (**c**) Plot of ln (C/Co) versus irradiation time for degradation kinetics of MB using different Ag NPs. Linear nature of the plots indicates a pseudo-first-order reaction (Aziz et al. 2015)

a challenge in itself and a useful model for other effluents and organic pollutants. For the purpose of comparison, a series of control experiments such as degradation in the absence of catalyst as well as degradation using commercial Ag NPs and bulk Ag powder were also performed under identical conditions. The changes in the optical absorption spectrum of MB dye at different time intervals are shown in Fig. 20.14a. In the absence of Ag NPs, the MB dye concentration remains almost unchanged after 30 min under visible light irradiation indicating that MB does not self-degrade under these conditions (Fig. 20.14b). The kinetics of MB degradation using different catalysts is presented in Fig. 20.14c. The observed kinetics was found to follow a pseudo-first-order reaction that can be modeled as

$$C_0/C = K_{\text{abs}}t \tag{20.1}$$

where K_{abs} is the apparent rate constant (min^{-1}), C_0 is the initial concentration of dye, and C is the concentration of dye at time t. It is worth noting that the dye concentration in these experiments remains in the optical regime where the Beer–Lambert law holds. The best-fit lines of $\ln(C_0/C)$ versus time during 0–150 min are plotted and presented in Fig. 20.14c. The near-linear nature of the data confirms that the photo-degradation of MB using the aforementioned catalysts follows pseudo-first-order kinetics. More importantly, the biosynthesized Ag NPs display superior photocatalytic activity towards the degradation of MB as compared to the commercially procured Ag NPs and bulk silver powder.

Another important real-world consideration for photocatalytic systems is its long-term stability. The biosynthesized Ag NPs were engaged to degrade MB dye for 150 min before the nanoparticles were removed and regenerated (heated to 100 °C for 60 min) and the process repeated for a total of five cycles. The biosynthesized Ag NPs were found to possess remarkable photostability with regard to photocatalytic degradation of MB dye, with less than 10 % decrease from its initial activity during the photodegradation.

20.6 Photocatalysis Mechanism

20.6.1 Cu-Doped ZnO Nanoparticles

The mechanism of photocatalysis for pure and Cu-doped ZnO nanorods has been illustrated in Fig. 20.15. ZnO has the source of electrons and holes for the degradation of organic dyes. The electron gets excited from the valence band to conduction band by absorbing UV light equal or higher band gap energy than energy band gap of ZnO. This leads to formation of holes in valence band with respect to the electrons excited to conduction Band (Zhang et al. 2014).

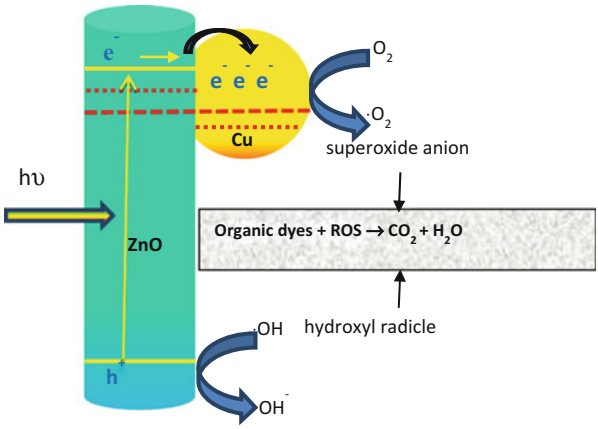

Fig. 20.15 The mechanism of photocatalysis for pure ZnO and Cu-doped ZnO nanorods (c.r.f. Bhuyan et al. 2015b)

$$\text{Zno} + h\nu \rightarrow e^{-}_{\text{CB}} + h^{+}_{\text{VB}}$$

The oxygen vacancy defects V_o^+ (Singly ionized oxygen defects), V_0^{++} (doubly charged state), and doped Cu nanoparticles on the surface of ZnO acts as sink in improving the generated electron–hole pair separation. The released electron from the Valence Band reacts with acceptors (like molecular oxygen) forming super oxide anion (Zhang et al. 2014; Leelavathi et al. 2013).

$$\text{V}_\text{o}^{++} + e^{-}_{\text{CB}} \rightarrow \text{V}_\text{o}^{+}$$
$$\text{V}_\text{o}^{+} + \text{O}_2 \rightarrow \text{V}_\text{o}^{++} + \text{O}_2^{-}$$
$$\text{Cu} \rightarrow \text{Cu}^{+2} + 2e^{-}(\text{or})\, e^{-}_{\text{CB}}$$
$$e^{-}(\text{or}) e^{-}_{\text{CB}} + \text{O}_2 \rightarrow \text{O}_2^{-}(\text{Super Oxide Anion})$$

Copper ion (Cu^{+2})even takes electrons from conduction band of ZnO decreasing the electron–hole pair recombination making more holes possible for OH radical formation.

$$2e^{-}_{\text{CB}} + \text{Cu}^{+2} \rightarrow \text{Cu}$$

The holes from valence band reacts with OH^- (moisture in air) forming OH (hydroxyl) radicals and reacts with H_2O forming OH radical and H^+ions

$$h^{+}_{\text{VB}} + \text{OH}^{-} \rightarrow \text{OH}'$$
$$h^{+}_{\text{VB}} + \text{H}_2\text{O} \rightarrow \text{OH}' + \text{H}^{+}$$

The generated O_2^- radical reacts with H_2O forming H_2O_2; this further reacts with electrons from conduction band forming more OHradicals (Arsana et al. 2012).

20.6.2 Silver Nanoparticles

The putative mechanism involved in the photocatalytic degradation of Ag NPs is diagrammatically represented in Fig. 20.16. Here, the photocatalytic reaction is initiated when a photoelectron is moved from the filled valence band of the Ag NPs to the empty conduction band upon visible light irradiation, as long as the irradiation energy ($h\nu$) is either equal to or greater than the band gap of Ag NPs. This leaves behind a hole in the valence band (h^+) and results in the formation of an electron (e^-)–hole pairs, which are powerful oxidizing and reducing agents, respectively (Zhan et al. 2011; Das et al. 2011). The high band gap of the nanoparticles leads to nonradiative recombination of electron and hole pairs (Danilczuk et al. 2006) that enhances the photocatalytic activity. Water, which is adsorbed on the

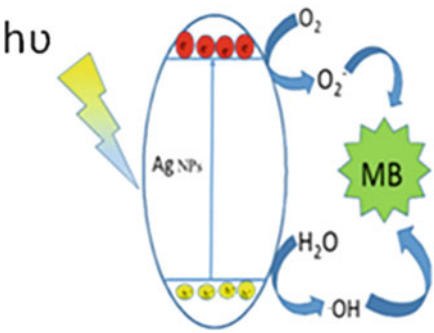

Fig. 20.16 Schematic mechanism for photocatalytic degradation of MB with biogenic Ag NPs (Aziz et al. 2015)

surface of Ag NPs, traps the hole and gets oxidized to give hydroxyl radical. Subsequently, electrons in the conduction band is taken up by oxygen, generating anionic superoxide radical which takes part in further oxidation process but also prevents the electron–hole recombination thus maintaining electron neutrality within the Ag NPs. The superoxide further combines with proton to give (HOO.) that ultimately generates H_2O_2, which in turn further dissociates into highly reactive hydroxyl radicals (HO.). These radicals such as HO. and $.O_2$- degrade MB by interacting with the aromatic ring of the MB and opening the azo bond and hydroxylated ring to yield CO_2, H_2O, SO_4^{2-}, NO^{3-}, and NH_4^+ ions (Brillas et al. 2009; Liu et al. 2007).

20.7 Conclusion

In the present work, the antibacterial and photocatalytic activity of nanoparticles was investigated. Engineered nanomaterials showed superior antibacterial action against Gram-positive as well as Gram-negative bacteria. The antibacterial activity of sample is elucidated using four main mechanisms: oxidative stress, coordination effect, non-homeostasis, and contact killing. In addition, these nanoparticles had shown better photocatalytic activity for degradation of organic water pollutants like methylene blue.

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References

Ameta A, Ameta R, Ahuja M (2013) Photocatalytic degradation of methylene blue over ferric tungstate. Sci Rev Chem Commun 3:172–180

Anselme K, Davidson P, Popa AM, Giazzon M, Liley M, Ploux L (2010) The interaction of cells and bacteria with surfaces structured at the nanometre scale. Acta Biomater 6:3824–3846

Armentano I, Dottori M, Fortunati E, Mattioli S, Kenny JM (2010) Biodegradable polymer matrix nanocomposites for tissue engineering: a review. Polym Degrad Stab 95:2126–2146

Arsana P, Bubpa C, Sang-Aroon W (2012) Photocatalytic activity under solar irradiation of silver and copper doped zinc oxide: photodeposition versus liquid impregnation methods. J Appl Sci 12:1809–1816

Arvizo RR, Bhattacharyya S, Kudgus RA, Giri K, Bhattacharya R, Mukherjee P (2012) Intrinsic therapeutic applications of noble metal nanoparticles: past, present and future. Chem Soc Rev 41:2943–2970

Aziz N, Faraz M, Pandey R, Shakir M, Fatma T, Varma A, Barman I, Prasad R (2015) Facile algae-derived route to biogenic silver nanoparticles: synthesis, antibacterial and photocatalytic properties. Langmuir 31:11605–11612

Baruah S, Sinha SS, Ghosh B, Pal SK, Raychaudhuri AK, Dutta J (2009) Photoreactivity of ZnO nanoparticles in visible light: effect of surface states on electron transfer reaction. J Appl Phys 105:074308. doi:10.1063/1.3100221

Bhuyan T, Mishra K, Khanuja M, Prasad R, Varma A (2015a) Biosynthesis of zinc oxide nanoparticles from *Azadirachta indica* for antibacterial and photocatalytic applications. Mater Sci Semicond Process 32:55–61

Bhuyan T, Khanuja M, Sharma R, Patel S, Reddy MR, Anand S, Varma A (2015b) A comparative study of pure and copper (Cu)-doped ZnO nanorods for antibacterial and photocatalytic applications with their mechanism of action. J Nanopart Res 17:288. doi:10.1007/s11051-015-3093-3

Brillas E, Sirés I, Oturan MA (2009) Electro-Fenton process and related electrochemical technologies based on Fenton's reaction chemistry. Chem Rev 109:6570–6631

Campoccia D, Montanaro L, Arciola CR (2013) A review of the biomaterials technologies for infection-resistant surfaces. Biomaterials 34:8533–8554

Chang YN, Zhang M, Xia L, Zhang J, Xing G (2012) The toxic effects and mechanisms of CuO and ZnO nanoparticles. Materials 5:2850–2871

Chen D, Qiao X, Qiu JC (2009) Synthesis and electrical properties of uniform silver nanoparticles for electronic applications. J Mater Sci 44:1076–1081

Danilczuk M, Lund A, Sadlo J, Yamada H, Michalik J (2006) Conduction electron spin resonance of small silver particles. Spectrochim Acta A Mol Biomol Spectrosc 63:189–191

Das DP, Biswal N, Martha S, Parida KM (2011) Solar-light induced photodegradation of organic pollutants over CdS-pillared Zirconium–Titanium Phosphate (ZTP). J Mol Catal A Chem 349:36–41

Fortunati E, Mattioli S, Visai L et al (2013) Combined effects of Ag nanoparticles and oxygen plasma treatment on PLGA morphological, chemical, and antibacterial properties. Biomacromolecules 14:626–636

Galhardi CM, Diniz YS, Rodrigues HG, Faine LA, Burneiko RC, Ribas BO, Novelli ELB (2005) Beneficial effects of dietary copper supplementation on serum lipids and antioxidant defenses in rats. Ann Nutr Metab 49:283–288

Huang Y-F, Chang H-T, Tan W (2008) Cancer cell targeting using multiple aptamers conjugated on nanorods. Anal Chem 80:567–572

Huanhuan L, Quansheng C, Jiewen Z, Khulal U (2015) Enhancing the antimicrobial activity of natural extraction using the synthetic ultrasmall metal nanoparticles. Sci Rep 5:11033

Kajbafvala A, Samberg JP, Ghorbani H, Kajbafvala E, Sadrnezhaad SK (2012) Effects of initial precursor and microwave irradiation on step-by-step synthesis of zinc oxide nanoarchitectures. Mater Lett 67:342–345

Leelavathi A, Madrasa G, Ravishankar N (2013) Origin of enhanced photocatalytic activity and photoconduction in high aspect ratio ZnO nanorods. Phys Chem Chem Phys 15:10795–10802

Linic S, Aslam U, Boerigter C, Morabito M (2015) Photochemical transformations on plasmonic metal nanoparticles. Nat Mater 14:567–576

Liu Z, Sun D, Guo P, Leckie JO (2007) One-step fabrication and high photo-catalytic activity of porous TiO_2 hollow aggregates by using a low-temperature hydrothermal method without templates. Chem Eur J 13:1851–1855
Macomber L, Imlay JA (2009) The iron-sulfur clusters of dehydratases are primary intracellular targets of copper toxicity. Proc Natl Acad USA 106:8344–8349
Mohan R, Krishnamoorthy K, Kim SJ (2012a) Diameter dependent photocatalytic activity of ZnO nanowires grown by vapor transport technique. Chem Phys Lett 539–540:83–88
Mohan R, Krishnamoorthy K, Kim SJ (2012b) Enhanced photocatalytic activity of Cu-doped ZnO nanorods. Solid State Commun 152:375–338
Moos PJ, Chung K, Woessner D, Honeggar M, Cutler NS, Veranth JM (2010) ZnO particulate matter requires cell contact for toxicity in human colon cancer cells. Chem Res Toxicol 23:733–739
Nel AE, Madler L, Velegol D, Xia T, Hoek EMV, Somasundaran P, Klaessig F, Castranova V, Thompson M (2009) Understanding biophysicochemical interactions at the nano-bio interface. Nat Mater 8:543–557
Pisanic TR, Jin S, Shubayev VI (2009) Nanotoxicity: from in vivo and in vitro models to health risks. Wiley, London, pp. 397–425
Prasad R (2014) Synthesis of silver nanoparticles in photosynthetic plants. J Nanopart 963961. doi:10.1155/2014/963961
Prasad R, Pandey R, Barman I (2015) Engineering tailored nanoparticles with microbes: quo vadis? WIREs Nanomed Nanobiotechnology 8:316–330
Rao L, Cui Q, Xu X (2010) Electronic properties and desolvation penalties of metal ions plus protein electrostatics dictate the metal binding affinity and selectivity in the copper efflux regulator. J Am Chem Soc 132:18092–18102
Raveendran P, Fu J, Wallen SL (2003) Completely green synthesis and stabilization of metal nanoparticles. J Am Chem Soc 125:13940–13941
Santo CE, Lam EW, Elowsky CG, Quaranta D, Domaille DW, Christopher CJ, Grass G (2011) Bacterial killing by dry metallic copper surface. Appl Environ Microbiol 77:794–802
Shamim N, Sharma VK (2013) Sustainable nanotechnology and the environment: advances and achievements. ACS Sym Ser Am Chem Soc 1124:165–179. doi:10.1021/bk-2013
Soenen SJH, Himmelreich U, Nuytten N, Pisanic TR, Ferrari A, De Cuyper M (2010) Intracellular nanoparticle coating stability determines nanoparticle diagnostics efficacy and cell functionality. Small 6:2136–2145
Srinivasa RB, Krishna V, Ravindhranath K (2012) Removal of methylene blue dye from waste waters using new bio-sorbents derived from *Annona squamosa* and *Azadiracta indica* plants. J Chem Pharm Res 4:4682–4694
Thakkar KN, Mhatre SS, Parikh RY (2010) Biological synthesis of metallic nanoparticles. Nanomedicine 6:257–262
Ueno T, Yokoi N, Abe S, Watanabe Y (2007) Crystal structure based design of functional metal/protein hybrids. J Inorg Biochem 101:1667–1675
Verma A, Uzun O, Hu Y, Hu Y, Han HS, Watson N, Chen S, Irvine DJ, Stellacci F (2008) Surface-structure regulated cell-membrane penetration by monolayer-protected nanoparticles. Nat Mater 7:588–595
Walch M, Dotiwala F, Mulik S, Thiery J, Kirchhausen T, Clayberger C, Krensky AM, Martinvalet D, Lieberman J (2014) Cytotoxic cells kill intracellular bacteria through granulysin-mediated delivery of granzymes. Cell 157:1309–1323
Wiley B, Sun Y, Xia Y (2007) Synthesis of silver nanostructures with controlled shapes and properties. Acc Chem Res 40:1067–1076
Xia T, Kovochich M, Liong M, Mädler L, Gilbert B, Shi H, Yeh JI, Zink JI, Nel AE (2008) Comparison of the mechanism of toxicity of zinc oxide and cerium oxide nanoparticles based on dissolution and oxidative stress properties. ACS Nano 2:2121–2134
Xie J, Lee JY, Wang DI, Ting YP (2007a) Identification of active biomolecules in the high yield synthesis of single-crystalline gold nanoplates in algal solutions. Small 3:668–672

Xie J, Lee JY, Wang DIC, Ting YP (2007b) Silver nanoplates: from biological to biomimetic synthesis. ACS Nano 1:429–439

Yu H, Ming H, Gong J, Li H, Huang H, Pan K, Liu Y, Kang Z, Wei J, Wang D (2013) Facile synthesis of Au/ZnO nanoparticles and their enhanced photocatalytic activity for hydroxylation of benzene. Bull Mater Sci 36:367–372

Zhan G, Du M, Sun D, Huang J, Yang X, Ma Y, Ibrahim A-R, Li Q (2011) Vapor-phase propylene epoxidation with H_2/O_2 over bioreduction Au/TS-1 catalysts: synthesis, characterization and optimization. Ind Eng Chem Res 50:9019–9026

Zhang X, Qin J, Xue Y, Yu P, Zhang B, Wang L, Liu R (2014) Effect of aspect ratio and surface defects on the photocatalytic activity of ZnO nanorods. Sci Rep 4:4596. doi:10.1038/srep04596

Chapter 21
Cryopreservation of Microorganisms

S.K. Singh and Abhishek Baghela

Abstract Microbes play vital roles in development of various disciplines of science and becoming basis of bio-economy. They are included in approximately 20 categories of biological matter specified in standard text relating to Convention on Biological Diversity (CBD). Conservation of these microorganisms has become a topic of great concern worldwide, and various methods are being practiced for long-term preservation and maintenance of these microorganisms to various purposes. Several methods with modifications in recipes have been in use. Though, no single method is complete for preserving all groups of microbes, their cryopreservation for long-term maintenance below –130 °C is generally regarded as safe, barring a few exceptions. However, success of cryopreservation depends on factors like type of materials, choice of the cryoprotectant, rates of cooling and thawing, etc. Preservation of cultures between –190 and 196 °C either in liquid or vapour phase (of liquid nitrogen) gives excellent results. This chapter deals with one of the most reliable methods applying cryogenic technique of long-term storage of microorganisms useful for small to large collections/bioresource centres, their advantages and disadvantages.

21.1 Introduction

On-going destruction of natural habitats necessitates development of alternative strategies for conserving our natural wealth. The conservation of microbial genetic resources by preserving live cultures in germplasm banks and dried/exsiccate specimens in mycological herbaria (Hawksworth 1974) have been traditional ways of long-term preservation. Conservation of microbial genetic resources on a sustained basis has become a strategic requirement for supporting basic and applied

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research. Standard text of CBD also includes microbes as biological matter (CGIAR 2001). Primary objectives of preserving and storing of microbial strains are intended to maintain organisms in a viable state to ensure their morphological, physiological, and genetic stability under laboratory conditions. This requires a thorough understanding of the physiology and nutritional requirements of individual strains. Rates of mitotic recombination and mutation that correspond to cell division are considered to influence metabolic activities of microbes (Jong and Birmingham 1992). Minor variations in culture conditions, environmental parameters, and subsequent techniques can result in larger variations of their metabolic profiles. Therefore, general laboratory/culture collections need to employ a standard mechanism to assess the effectiveness of preservation method (s) over metabolic integrity of each and every strain, as a quality control measure. This is accomplished by storage of microbial germplasm using various preservation methods in practice that range from continual subculturing to the method of halting/suspending the metabolism of particular strains under subzero temperature. Survival ability of living organisms to freezing and thawing was realised for the first time in 1663 when nematodes were frozen and revived (Morris 1981).

Though no single method is ideal for preserving all groups of microorganisms including fungi, their maintenance under subzero temperature is generally regarded as safe and reliable method, but with some exceptions. Most sporulating and non-sporulating fungi that grow well in culture survive cryopreservation. However, success of this method depends on several factors. The critical periods in cryopreservation are during freezing and thawing processes. Principally, two types of freezing protocols are recognized: a slow or controlled one and fast or uncontrolled one. Both have been used, and their positive and negative impacts have been reported from time to time. Generally, too low freezing rates are reported to cause excessive dehydration, ultimately leading to cells damage; on the contrary, too fast freezing leads to insufficient dehydration and formation of abundant ice crystals having lethal consequences. In general, best results in the survival and recovery of most microbial cultures have been obtained using a slow cooling rate and rapid thawing.

21.2 Principle of Cryoprotection

In principle, cryopreservation is the use of ultra-low temperature to preserve intact living cells and tissues. The cooling effect is important phenomenon, dominated by the freezing of water, which results in the concentration of the solutes that are dissolved in the remaining liquid phase. Incidence of freezing injury has been a great concern, and general opinion has been developed that intracellular ice formation is harmful, whereas extracellular freezing is comparatively harmless. It has been reported that ice crystals pierce or tease through the cells, destroying

them directly or indirectly *via* changes in the composition of the liquid phase (Pegg 2007). Cooling rates, in this regard, play vital role on cell survival. The extracellular ice formation some times becomes harmful as densely packed cells are prone to damage by mechanical stresses within the channels where they are sequestered. It is, however, advised to secure cell survival and to avoid damage to the extracellular complex structure. Ice formation can be avoided up to some extent by vitrification, but toxicity has the major concern. In view of these facts, always there is a need of improving and optimizing preservation protocol, as single method may not be applicable to all types of microbes. In case of fungi, it has been observed that several strains of fungi show attenuation in their growth when grown on solid nutrient media. In extreme conditions, it can result in total loss.

Cryoprotection involves treatment of the cells or tissues to be preserved with cryoprotectant solutes. In variable concentration, these solutes are considered as driving force of movement of water by osmosis and of solutes by diffusion. There are numerous barriers to the free diffusion of solutes which can result in transient, and sometimes equilibrium, changes in compartment volumes, which can be lethal. Therefore, diffusion and osmosis phenomena are important and play vital roles in cryoprotection and found widespread application in biology.

21.3 Use of Cryoprotectant

Report states that effectiveness of cryopreservation of microbes depends on species, strain, cell size and form, growth phase and rate, incubation temperature, growth medium composition, pH, osmolarity and aeration, cell water content, lipid content and composition of the cells, density at freezing, composition of the freezing medium, cooling rate, storage temperature and duration of storage, warming rate, and recovery medium (Ashwood-Smith 1980; Calcott 1978; Day and McLellan 1995; Heckly 1978; Hubalek 1996; Kirsop and Doyle 1991; Kirsop and Snell 1984). One of the most important conditions is the composition of the medium used for suspending organisms for freezing. Freezing damage, however, has been a matter of great concern (Lovelock 1953; Polge et al. 1949) and has led to the development of modern cryotechnology. However, freezing damage can be avoided by using some compounds termed as cryoprotectant sometimes referred to as cryoprotective agent (CPA).

Cryoprotectant can be any additive, provided to cells before freezing, which yields a higher post-thaw survival. Although a good survival of deep-frozen microbes has occasionally been observed without a protective additive, the presence of a suitable CPA is reported to increase the survival considerably. Such additives simply increase total concentration of solutes in the system and

substantially reduce the amounts of ice formation at any given temperature, but in principle to be biologically acceptable, they must be able to penetrate the cells and have low toxicity. Literature indicates that many compounds are reported to have such properties and being practiced in many laboratories and culture collections. These CPA can be classified in various ways, such as either low-MW or high-MW additives (Nash 1966), depending upon the rate of penetration, based on penetrating and non-penetrating cell wall and/or cell membrane. Thus, three categories of additive might be distinguished (Tao and Li 1986):

(A) Cryoprotectants penetrating cell wall and cell membrane
(B) Cryoprotectants penetrating cell wall but not cell membrane
(C) Cryoprotectants neither penetrating cell wall nor cell membrane

Polge et al. (1949) reported successful preservation of avian spermatozoa using 10–20 % of glycerol at –80 °C. Permeability of some of these solutes (e.g., glycerol) depends on temperature and cell type, and some penetrating CPAs might be regarded as low-permeable compounds under some circumstances. However, other CPAs penetrate only the cell wall (CW) and not the cytoplasmic membrane (CM). The penetrating CPAs usually include glycerol, methanol, ethanol, ethylene glycol (EG), propylene glycol (PG), dimethylformamide, methylacetamide, Me_2SO, etc. Sucrose; lactose; glucose; mannitol; sorbitol; dextran; mono-, oligo-, and polysaccharides; hydroxyethyl starch (HES); methyl cellulose; albumin; gelatin; other proteins; polyvinylpyrrolidone (PVP); polyethylene glycol (PEG); polyethylene oxide (PEO); or polyvinyl alcohol are non-penetrating or nonpermeating compounds (Jong and Birmingham 1992) that cause extracellular cryoprotection when present at concentrations of 10–40 %. Among these CPAs, widely used are glycerol and Me_2SO. Glycerol has been frequently used in cryopreservation of fungi while Me_2SO is used for algae and protozoa. Similarly, methanol has been widely used for the preservation of algae. Skimmed milk is the preferred CPA for bacteria.

21.4 Removal of Cryoprotectants

Removal of CPA is an important step in process which can be achieved by exposing the cells to a lower concentration of the compound. The mechanism of osmotic uptake of water causes cells to swell or reach the final volume, and then shrink as CPA moves out with sufficient water to maintain osmotic equilibrium. Therefore, it has been advised that rate of change of volume and the final volume must be considered while standardizing protocols of recovering cryopreserved cells/cultures (Pegg 2007).

21.5 Adaptability of Microbes to Cryopreservation

ATCC implemented cryopreservation method in 1960 in large numbers of fungal strain/species (Hwang 1960; Smith 1983). Later on, it was introduced to many other collections, e.g., CAB International Mycological Institute (Onions 1971), IFO—Institute for Fermentation Osaka (Ito 1996), etc. Subsequently, this method has been successfully used for other group of microorganisms. Feltham et al. (1978) reported use of glass beads at −76 °C for preservation of bacteria. The success of this method depends on adaptability of different microbes to certain conditions like specific temperature and CPA, etc.; therefore, adaptability of filamentous fungi, yeast, and bacteria is provided here in brief.

Filamentous Fungi Preservation of filamentous fungi at the ultra-low temperature of –196 °C in liquid or vapor phase has been regarded as safe and one of the best methods (Smith et al. 2001; Kirsop and Doyle 1991). With adequate care of freezing and thawing, any phenotypic or genotypic changes can be avoided. Optimization of the method for target fungal strain (s) has enabled successful freezing of many recalcitrant fungi (Morris et al. 1988b). Cryopreservation of fungi is in practice of many leading culture collections in the world as one of the most reliable methods (Homolka 2013). Literature indicates that in beginning researchers tried different options to cryopreserve spore suspension, mycelial/vegetative culture, separately as well as in combinations, with or without a CPA, and from time to time required improvements have been made in the method/recipe (Hwang 1968; Stalpers et al. 1987; Chandler 1994). Recently, Homolka et al. (2001, 2006) devised a method using perlite (aluminosilicate volcanic mineral) as a carrier for fungal mycelia and successfully tested for preserving basidiomycete strains. As most preferred condition, a cooling rate of –1 °C min^{-1} with use of 10 % (v/v) glycerol as a CPA is applied and reported to have good impact on major group of fungi. The same protocol is followed in the author's laboratory for cryopreservation of fungi. However, there have been reports on some members of the Basidiomycota and Oomycota that they do not survive cryopreservation well compared to sporulating fungi. Hence, species-specific standardization of cooling rates and use of CPA may provide improved viability. Therefore, finding the optimum cooling rate and choice of cryoprotectant (s) has always been the subject of intense research (Smith 1993; Hwang 1966; Morris et al. 1988b; Smith and Thomas 1998). Choice of cryoprotectant is a matter of experience which varies according to the organism. Glycerol is considered to provide satisfactory results. Dimethyl sulfoxide is often more satisfactory (Lovelock and Bishop 1959; Hwang and Howells 1968; Hwang et al. 1976), but is often toxic to sensitive organisms. Sugars and large molecular substances such as polyvinylpyrrolidine (Ashwood-Smith and Warby 1971; Smith 1983) have been used, but are less effective hence least preferred.

21.6 Recalcitrant Fungi and Their Preservation

Fragmentary report reveals nonconventional uses of cryotechnique, which include preservation of fungal species with their host. Fungi such as unculturable, obligate parasites, mutualists, etc., that are difficult to maintain in a living state can be preserved using this method. Some important recalcitrant fungi, viz., *Halophythophthora*, *Saprolegnia*, and *Aphanomyces* spp., microcylic rust fungi, some members of *Basidiomycota*, and the *Glomeromycota* can be preserved. Techniques used to preserve these fungal taxa include encapsulation and vitrification (Ryan and Smith 2004; Benson et al. 1996; Benson 1994). The use of encapsulation (immobilization) cryopreservation (Benson et al. 1996), involving entrapment of mycelium/spores in calcium alginate beads prior to preservation, is well documented, for example, *Serpula lacrymans* (Ryan 2001) and monoxenically produced spores of *Glomeromycota* (Declerck and Coppenolle 2000). In vitrification highly concentrated CPA solutions are applied to organisms of different cell types, especially plant cells, and it does not require controlled cooling. Samples are plunge cooled, with the vitrification solution that prevents onset of concentration effects/ or ice damage. For resuscitation from frozen state, care is taken to ensure that samples do not 'crack', which otherwise could cause physical damage to the mycelium. Samples must be immediately washed to remove the vitrification solution, as it is toxic to the fungi. Number of fungi has been preserved by this technique (Ryan and Smith 2004), though routine use of this technique is not advised due to toxicity. Both the techniques show significant potential for preserving recalcitrant fungi that could otherwise not be stored on long-term basis. However, the use of these techniques (for fungi) is still under active investigation and therefore not broadly tested, leaving ample scope to standardize the protocol for different group of recalcitrant fungi.

21.6.1 Yeast

Compared to the filamentous fungi, cryopreservation of yeast can be achieved by making suspension in glass ampoules, mostly in the liquid phase of the nitrogen. However, literature also suggests alternative method of storing yeast suspensions in vapor phase of the liquid nitrogen. The later method has been considered to provide security and extra protection against leakage of liquid nitrogen into the ampoules and contamination, and it saves storage space (Gilmour et al. 1978). This method was for the first time used for artificial insemination. Modification and refinement in principal method of cryopreservation have been continuing; therefore, variations in protocol and recipe are prevalent. Several yeast genera are reported to have low survival levels with difficulty in successfully freeze-drying,

including *Lipomyces*, *Leucosporidium*, *Rhodosporidium*, etc. The % viability in liquid phase has been reported more (more than 30 %) than in vapor phase. However, certain improvements in protocol have been tried by the researchers, viz., several culture collections uses trehalose as a protectant (Berny and Hennebert 1991; Roser 1991), and certain changes occur in the cells and their immediate environment (Pearson et al. 1990). Extracellular ice formation has been reported as the cells lead to dehydration and shrinkage (Diller and Knox 1983; Morris et al. 1988a). Cell shrinkage during freezing is considered very important phenomenon to prevent cellular damage, highly affected by cooling rate. During rapid cooling, cell gets insufficient time to dehydrate leading to intracellular ice formation causing damage to cell organelles (Morris et al. 1988a). There are reports indicating occurrence of genetic damage, if the nucleus disrupted (Pearson et al. 1990; Calcott et al. 1983).

21.6.2 Bacteria

Cryopreservation of bacteria is not routinely undertaken in some collections, while others use this method for bacteria, especially which do not survive freeze-drying. The glass beads method has been used in a few collections for years together. Except certain obligate methylotrophic bacteria that were found to lose viability during storage over liquid nitrogen, no problems have been encountered with most bacterial genera.

21.7 Protocols

Considering reliability of cryopreservation for long-term storage of important microorganisms, modifications in different protocols have been reported (Kirsop and Snell 1984; Smith and Onions 1980, 1983; Elliott 1976; Stalpers et al. 1987) in order to make it suitable with available equipment, preference of materials, or the type of organism. Graphical presentations (flow chart) of a simplified protocol are provided at the end (Boxes 21.1 and 21.2), which are applicable to cryopreservation of filamentous fungi (Nakasone et al. 2004; with some modifications), yeasts (Bond 2007), and bacteria (Hoffmann 1989; Malik and Claus 1987). Protocol provided for fungi is also being practiced for conserving fungi at National Fungal Culture Collection of India (NFCCI-WDCM 932).

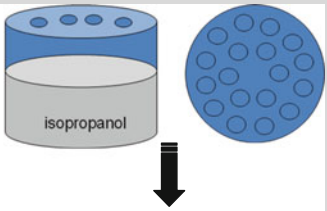
Box 21.1

Protocol for preservation of fungal cultures in liquid nitrogen (CRYOPRESERVATION)

Cultures are grown on PDA or MEA plates

Quality check is done by slide preparation and microscopy

Plugs are cut with flame sterilized cork borer & aseptically transferred to cryovials containing 10% glycerol labelled using cryomarker

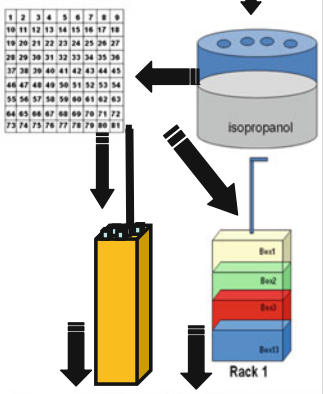


Place the tightly capped cryovials in Nalgene freeze containers filled with isopropanol

-70°C Deep Freezer

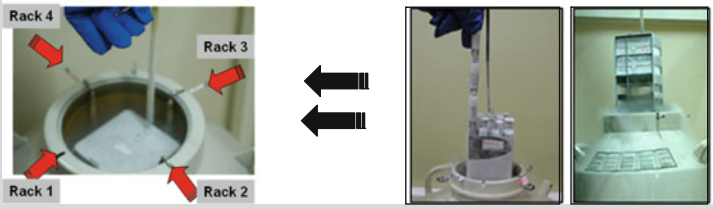
Nalgene ® freeze containers are kept in -70°C deep freezer for 4 hrs.

It allows freezing of samples with nearly 1°C/min cooling rate

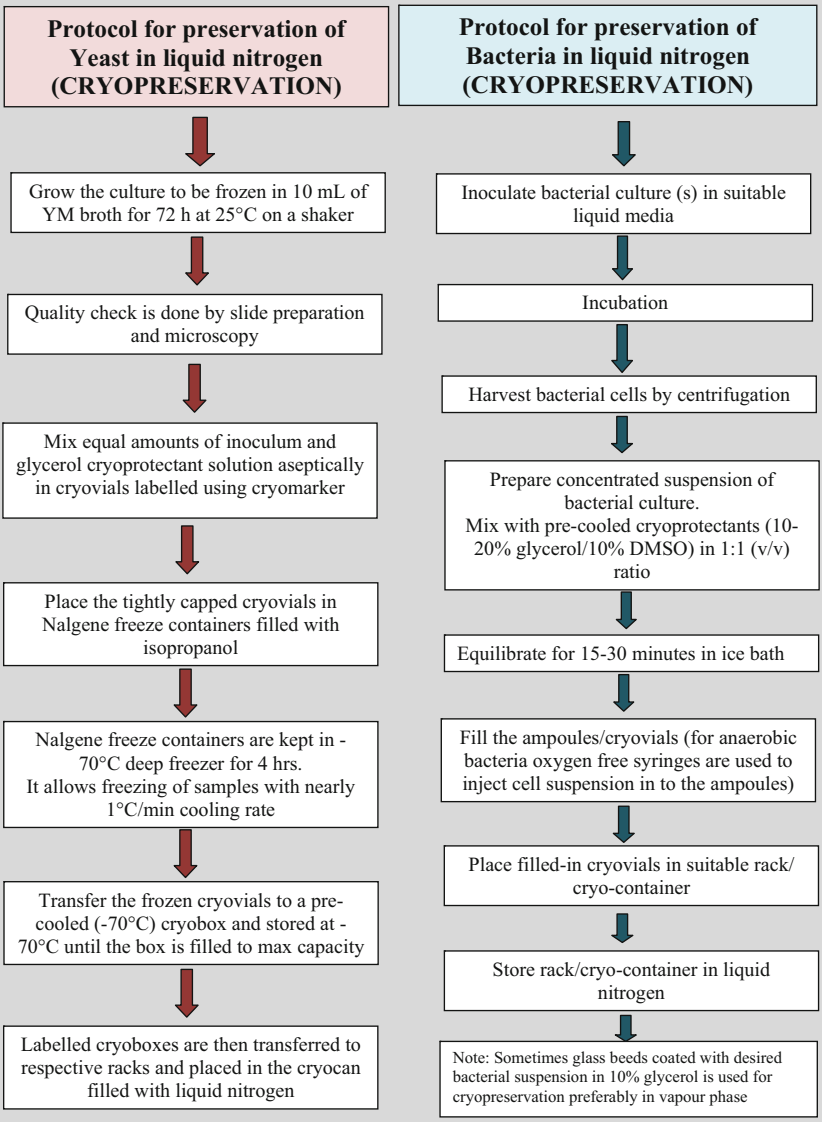


Transfer the frozen cryovials to pre-cooled (-70°C) cryoboxes and stored at -70°C until the box is filled to max capacity

Labelled cryoboxes and/or cryocanes are then transferred to respective racks and placed in the cryocan filled with liquid nitrogen



Box 21.2


Protocol for preservation of Yeast in liquid nitrogen (CRYOPRESERVATION)
Grow the culture to be frozen in 10 mL of YM broth for 72 h at 25°C on a shaker
Quality check is done by slide preparation and microscopy
Mix equal amounts of inoculum and glycerol cryoprotectant solution aseptically in cryovials labelled using cryomarker
Place the tightly capped cryovials in Nalgene freeze containers filled with isopropanol
Nalgene freeze containers are kept in -70°C deep freezer for 4 hrs. It allows freezing of samples with nearly 1°C/min cooling rate
Transfer the frozen cryovials to a pre-cooled (-70°C) cryobox and stored at -70°C until the box is filled to max capacity
Labelled cryoboxes are then transferred to respective racks and placed in the cryocan filled with liquid nitrogen
Protocol for preservation of Bacteria in liquid nitrogen (CRYOPRESERVATION)
Inoculate bacterial culture (s) in suitable liquid media
Incubation
Harvest bacterial cells by centrifugation
Prepare concentrated suspension of bacterial culture. Mix with pre-cooled cryoprotectants (10-20% glycerol/10% DMSO) in 1:1 (v/v) ratio
Equilibrate for 15-30 minutes in ice bath
Fill the ampoules/cryovials (for anaerobic bacteria oxygen free syringes are used to inject cell suspension in to the ampoules)
Place filled-in cryovials in suitable rack/ cryo-container
Store rack/cryo-container in liquid nitrogen
Note: Sometimes glass beeds coated with desired bacterial suspension in 10% glycerol is used for cryopreservation preferably in vapour phase

21.8 Viability Testing

It is always advised to record observation on viable cells before and after applying any preservation methods for comparative analysis and consideration of its suitability. There may be different ways to take observations. The simplest method is to examine cells microscopically in a counting chamber using vital stains. Other most common way to take this observation is to add 1 mL of original cell suspension to 9 mL of sterile distilled water. After making further dilutions upto10^{-6}, transfer 0.5 mL from each of dilutions of 10^{-6}–10^{-3} onto suitable agar/nutrient medium. In case of filamentous fungi, PDA may be used as general medium. After incubating the inoculation plates at optimal temperatures recommended for a specific group of organism, viz., 25 °C for 72 h, or longer for fungi, observations are recoded as cell counts. After preservation, recovered viable cells were counted by adding two drops of the thawed cell suspension (0.06 mL) to 0.54 mL of sterile, distilled water. From this stock (10^{-1} dilution), additional dilutions up to 10^{-6} dilutions may be prepared, then inoculate 0.5 mL suspension from desired dilution on suitable agar/nutrient medium plates, incubate at optimal temperature recommended for a particular group of fungi, and record the cell counts. The percentage viability of the culture is calculated and recorded.

21.9 Growth, Morphology and Inventory

After viability testing, the selected strain (s) is grown to its optimal conditions and, if required, on different agar media in order to check overall growth behaviors and comparative analysis of pre-freezing morphological characteristics with post freezing characteristics. Any changes in morphology and growth behaviors are recoded, and data are stored safely for future comparative analysis.

Making inventory of cryopreserved samples is an important task required by culture collections for easy storage and retrieval of strains using different types of storage tanks. Considering sensitivity of the method, it is generally advised to maintain strains at least in duplicate sets separately in two storage tanks. It sometimes depends on the size of the collection. Since different types and ranges of materials used in cryopreservation are available like storage boxes, canisters, racks, straw, and cyovials, a suitable and easy to use inventory is necessary to prepare and maintain as per requirement by the individual collection.

21.10 Advantage and Disadvantage

Storage of cultures in liquid nitrogen, although technically simple, can involve relatively high running costs because of the necessity of regular filling of the containers with liquid nitrogen. The initial cost of the equipment is comparable to

that used for freeze-dying, but the costs and problems associated with the handling of liquid nitrogen have led some collections to seek alternatives. However, for most workers, the technique of liquid nitrogen storage is convenient, well tried, tested and unlikely to be superseded in the near future.

21.11 Common Considerations/Important Tips!!

- Use of polystyrene foam box provides insulation for 1 °C/minute cooling
- Use standard cryovials
- Polypropylene cryovials are resistant to cracking at ultralow temperature.
- Some repositories prefer glass cryovials
- Use an alcohol resistant cryomarker for proper labeling
- Use standard cryolebel
- Size of agar plugs should be less than diameter of mouth of cryovials
- Prior to opening, for revival of preserved strain (s), disinfect the surface of cryovials by dipping in alcohol.

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References

Ashwood-Smith MJ (1980) Preservation of microorganisms by freezing, freeze-drying and desiccation. In: Ashwood-Smith MJ, Farrant J (eds) Low temperature preservation in medicine and biology. Pitman Medical, Tunbridge Wells, pp 219–252

Ashwood-Smith MJ, Warby C (1971) Studies on the molecular weight and cryoprotective properties of PVP and Dextran. Cryobiology 8:453–464

Benson EE (1994) Cryopreservation. In: Dixon RA (ed) Practical plant cell culture: a practical approach. IRL, Oxford, UK, pp 147–166

Benson EE, Wilkinson M, Todd A, Ekuere U, Lyon J (1996) Developmental competence and ploidy stability in plants regenerated from cryopreserved potato shoot tips. CryoLetters 17:119–128

Berny JF, Hennebert GL (1991) Viability and stability of yeast cells and filamentous fungus spores during freeze-drying—effects of protectants and cooling rates. Mycologia 83:805–815

Bond C (2007) Cryopreservation of yeast cultures. Cryopreservation and freeze-drying protocols. Methods Mol Biol 368:109–117

Calcott PH (1978) Freezing and thawing microbes. Meadow Field, Durham

Calcott P, Wood D, Anderson L (1983) Freezing and thawing induced curing of drug-resistance plasmids from bacteria. CryoLetters 4:99–106

CGIAR (2001) CGIAR working document on IP. Report from mid-term meeting, 21–25 May 2001, Durban, South Africa. Consultative Group on International Agricultural Research, Washington, DC

Chandler D (1994) Cryopreservation of fungal spores using porous beads. Mycol Res 98:525–526
Day JG, McLellan MR (1995) Cryopreservation and freeze-drying protocols, Methods in molecular biology, vol 38. Humana, Totowa, NJ
Declerck S, Coppenolle MGAV (2000) Cryopreservation of entrapped monoxenically produced spores of an arbuscular mycorrhizal fungus. New Phytol 148:169–176
Diller KR, Knox JM (1983) Automated computer-analysis of cell-size changes during cryomicroscope freezing—a biased trident convolution mask technique. CryoLetters 4:77–92
Elliott TJ (1976) Alternative ampoule for storing fungal cultures in liquid nitrogen. Trans Br Mycol Soc 67:545–546
Feltham RKA, Power AK, Pell PA, Sneath PHA (1978) A simple method for storage of bacteria at –76°C. J Appl Bacteriol 44:313–316
Gilmour MN, Turner G, Berman RG, Krenzer AK (1978) Compact liquid nitrogen storage system yielding high recoveries of gram-negative anaerobes. Appl Environ Microbiol 35:84–88
Hawksworth DL (1974) Mycologist's handbook. Commonwealth Agricultural Bureau, Farnham Royal, Slough 213 pp
Heckly RJ (1978) Preservation of microorganisms. Adv Appl Microbiol 24:1–53
Hoffmann P (1989) Cryopreservation of fungi. World Federation for Culture Collections. UNESCO/WFCC-Education Committee, Braunschweig, Germany. Technical Information Sheet No. 5
Homolka L (2013) Methods in cryopreservation in fungi. In: Gupta VK, Tuohy MG, Ayyachamy M, Turner KM, Donovan AO (eds) Laboratory protocols in fungal biology. Springer, New York, pp 9–16
Homolka L, Lisa L, Eichlerova I, Nerud F (2001) Cryopreservation of basidiomycete strains using perlite. J Microbiol Methods 47:307–313
Homolka L, Lisa L, Nerud F (2006) Basidiomycete cryopreservation on perlite, evaluation of a new method. Cryobiology 52:446–453
Hubalek Z (1996) Cryopreservation of microorganisms at ultra-low temperatures. Academia, Prague
Hwang S-W (1960) Effects of ultralow temperature on the viability of selected fungus strains. Mycologia 52:527–529
Hwang S-W (1966) Long term preservation of fungus cultures with liquid nitrogen refrigeration. Appl Microbiol 14:784–788
Hwang S-W (1968) Investigation of ultra-low temperature for fungal cultures. I. An evaluation of liquid nitrogen storage for preservation of selected fungal cultures. Mycologia 60:613–621
Hwang S-W, Howells A (1968) Investigation of ultra-low temperature of fungal cultures. II. Cryoprotection afforded by glycerol and dimethyl sulfoxide to eight selected fungal cultures. Mycologia 60:622–626
Hwang S-W, Kylix WF, Haynes WC (1976) Investigation of ultra low temperature for fungal cultures III. Viability and growth rate of mycelial cultures cryogenic storage. Mycologia 68:377–387
Ito T (1996) Preservation of fungal cultures at the Institute of Fermentation, Osaka (IFO). In: Samson RA, Stalpers JA, van der Mei D, Stouthamer AH (eds) Culture collections to improve the quality of life. The Netherlands and the World Federation for Culture Collections, Centraalbureau voor Schimmelcultures, Baarn, pp 210–211
Jong SC, Birmingham JM (1992) Current status of fungal culture collections and their role in biotechnology. In: Arora DK, Elander RP, Mukerji KG (eds) Handbook of applied mycology. Marcel Deker, New York, pp 993–1024
Kirsop BE, Doyle A (1991) Maintenance of microorganisms and cultured cells: a manual of laboratory methods. Academic press, London
Kirsop BE, Snell JJS (eds) (1984) Maintenance of microorganisms: a manual of laboratory methods. Academic press, New York 207 pp
Lovelock JE (1953) The mechanism of the protective action of glycerol against haemolysis by freezing and thawing. Biochim Biophys Acta 11:28–36

Lovelock JE, Bishop MWH (1959) Prevention of freezing damage to living cells by dimethylsulphoxide. Nature 183:1394–1395
Malik KA, Claus D (1987) Bacterial culture collections: their importance to biotechnology and microbiology. Biotechnol Genet Eng Rev 5:137–197
Morris GJ (1981) Cryopreservation: An introduction to cryopreservation in culture collections, Cambridge Culture Centre of Algae and Protozoa. Institute of Terrestrial Ecology, Cambridge
Morris GJ, Coulson GE, Clarke KJ (1988a) Freezing injury in *Saccharomyces cerevisiae*—the effect of growth conditions. Cryobiology 25:471–482
Morris GJ, Smith D, Coulson GE (1988b) A comparative study of the morphology of hyphae during freezing with the viability upon thawing of 20 species of fungi. J Gen Microbiol 134:2897–2906
Nakasone KK, Peterson SW, Jong S-C (2004) Preservation and distribution of fungal cultures. In: Mueller GM, Bills GF, Foster MS (eds) Biodiversity of fungi: inventory and monitoring methods. Elsevier Academic, Amsterdam, pp 37–47
Nash T (1966) Chemical constitution and physical properties of compounds able to protect living cells against damage due to freezing and thawing. In: Meryman HT (ed) Cryobiology. Academic press, London, pp 179–211
Onions AHS (1971) Preservation of fungi. In: Booth C (ed) Methods in microbiology, vol 4. Academic press, London, pp 113–115
Pearson BM, Jackman PJH, Painting KA, Morris GJ (1990) Stability of genetically manipulated yeasts under different cryopreservation regimes. CryoLetters 11:205–210
Pegg DE (2007) Principles of cryopreservation. In: Day JG, Stacey GN (eds) Methods in molecular biology, vol. 368: Cryopreservation and freeze-drying protocols, 2 edn. Humana, Totowa, NJ, pp 39–57
Polge C, Smith AU, Parkes AS (1949) Revival of spermatozoa after vitrification and dehydration at low temperatures. Nature 164:666. doi:10.1038/164666a0
Roser B (1991) Trehalose drying: a novel replacement for freeze drying. Biopharm 4:47–53
Ryan MJ (2001) The use of immobilisation for the preservation of *Serpula lacrymans*. Mycologist 15:66–69
Ryan MJ, Smith D (2004) Fungal genetic resource centres and the genomic challenge. Mycol Res 108:1351–1362
Smith D (1983) Cryoprotectants and the cryopreservation of fungi. Trans Br Mycol Soc 80:360–363
Smith D (1993) Tolerance to freezing and thawing. In: Jennings DH (ed) Stress tolerance of fungi. Marcel Dekker, New York, pp 145–171
Smith D, Onions AHS (1980) The preservation and maintenance of living fungi. Commonwealth Mycological Institute, Kew, UK
Smith D, Onions AHS (1983) A comparison of some preservation techniques for fungi. Trans Br Mycol Soc 81:535–540
Smith D, Thomas VE (1998) Cryogenic light microscopy and the development of cooling protocols for the cryopreservation of filamentous fungi. WJMB 14:49–57
Smith D, Ryan MJ, Day JG (2001) The UKNCC biological resource: properties, maintenance, and management. UKNCC Secretariat, Egham, Surrey, UK
Stalpers JA, de Hoog A, Vlug IJ (1987) Improvement of the straw technique for the preservation of fungi in liquid nitrogen. Mycologia 79:82–89
Tao D, Li PH (1986) Classification of plant cell cryoprotectants. J Theor Biol 123:305–331

Chapter 22
Microbial Cultures: Maintenance, Preservation and Registration

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Abstract Microorganisms account for major biomass on the Earth and play an important role in biogeochemical processes that are important for survival of all forms of life on this planet. In this context, the conservation of microbial communities and their ecosystem should be given high priority. Microbiologists have always thought of preservation of culturable microbial diversity in adequate manner without changes in morphological, physiological, and genetic traits. Culture collections (ex situ conservation), also known as microbial resource centres (MRCs), have made huge contributions in storage and preservation of all kinds of microorganisms. In this chapter, we have discussed ex situ preservation methods of microorganisms followed in National Agriculturally Important Microbial Culture Collection (NAIMCC) and other culture collections across the world. Four international culture collections and seventeen (17) national culture collections having either status of International Depository Authority (IDA) or National Biodiversity Authority (NBA) or both have been discussed in this chapter. This chapter also highlights short-, medium-, and long- term methods used for the preservation of microorganisms along with some latest techniques such as Sordelli's method, vitrification, and encapsulation. Some critical factors that affect the cell survival and recovery during the process of preservation have also been incorporated. In general, description of NAIMCC with its infrastructure and activities including registration of elite microbial germplasm has been highlighted for benefits of researchers, farmers, and industries of India and abroad.

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22.1 Introduction

Microorganisms, the oldest form of life on earth, constitute a total number of 5×10^{30} cells which contribute a biomass of 104.9×10^{15} g of carbon (Scharf 2009). Microorganisms, including bacteria, archaea, fungi, and protists, are so tiny that they cannot be seen by the naked eye. Archaea are bacteria-like creatures that have some specific traits not found in any true bacteria. Protists include primitive algae, amoebas, slime moulds, and protozoa. Viruses are considered to be on the borderline between living organisms and nonliving things. They are abundant in nature, have plasticity in their genomes to adapt to varying environmental conditions, and have diversity in their mode of nutrition. In the lithosphere, microbes possess the remarkable property to utilize both organic and inorganic substances as nutrient for their growth and development. They occupy an important place in every walk of human life. In the air we breathe, the surface on which we walk, the food we eat, everywhere in our surroundings, as well as inside our body, trillions of microorganism are present. A spoonful of garden soil contains 1,000,000,000 (10^9) bacteria, 120,000 (1.2×10^5) fungi, and 25,000 (2.5×10^4) algae of more than hundred types. They have become so innate part of our system that life without them seems to be impossible. Plants will fail to grow in spite being provided with nutrients and stinking waste of our homes will not decay and will be left out with less oxygen to breathe. Microbial decomposition is important in maintaining the balance of nature. Without decomposition by microorganisms, the cycling of important elements like carbon, nitrogen, sulphur, iron, oxygen, and hydrogen would be impossible. There are many such activities to count which shows microbes are vital for life on earth.

Some microorganisms are our friends and others are foes. They are often referred to as useful microorganisms and harmful microorganisms. Useful microorganisms are non-pathogenic and produce wonder drugs that control various ailments and useful products like enzymes, amino acid, vitamins, alcohol, etc. Microbial nitrogen fixation process fixes 255×10^6 metric tonnes nitrogen each year utilizing free atmosphere nitrogen (Shridhar 2012). Microorganisms are involved in nutrient flux in the biosphere by participating in various processes such as rock weathering, organic decomposition, and transformations of a range of essential elements. Harmful microorganisms that cause deadly diseases in plants, animals, and humans are often referred as pathogens.

Microorganisms are responsible for existence of life on the Earth by their contribution in various ways. Realizing the potential of microorganisms and loss of biodiversity under the changing climatic conditions, many countries have developed repository and begun conservation of microbial wealth of the world for their utilization in agriculture and industry for fulfilling the needs of society.

22.2 Microbial Conservation and Preservation

Conservation is the act of preserving, guarding, or protecting. The objective is to protect microorganisms from being lost or damaged from something which can harm it. Conservation maintains the quality constant throughout the physical and chemical reactions or evolutionary changes. Microorganisms are dominating life on Earth; these are the only biological entities that persist in the deep subsurface, where multicellular organisms cannot persist. Microorganisms play an important role in forming the base of food chain, biogeochemical transformation, biosphere health, and ecosystem. In addition, microbial diversity has huge resources of value for the pharmaceutical, biotechnology, and food industries. The loss of microbial diversity involves a loss of potentially valuable resources on which human population is dependent. However, the attention toward microorganism's conservations has not received much impetus. One reason to conserve and protect microorganisms is that our survival depends on them. A challenge for microbial conservation is to conserve individual species in the natural environment. Individual species or strains can be preserved by isolation and culturing ex situ in culture collections. Another challenge is the protection of ecosystem diversity. Microorganisms are evolving rapidly and have a tendency to exchange genetic information. Prakash et al. (2013) have suggested that the protection of large areas of a habitat is required to maximize the conservation of the microbial diversity. There are two types of strategies (in situ conservation and ex situ preservation) that help to improve the incorporation of microbial communities and ecosystems into conservation agenda. Isolation, handling and preservation of microorganisms in the culture collections is known as ex situ preservation which is widely being followed throughout world.

22.2.1 In Situ *Conservation*

In situ conservation includes on-site conservation of the microbial flora along with their ecosystem and habitat and the maintenance as well as recovery. Conservation of all subsets of life existing in interplaying networks leads to enhanced necessity for preservation of microbes. Microbes loose important traits with time either by mutation or loss of certain genes. They comprise the greatest numbers of individual organisms on earth and only small fractions of them are known to us (Cockell and Jones 2009; Olembo and Hawksworth 1991). Most of them cannot be cultured using currently available techniques, and thereby their conservation in their niche is utmost important. Torrential rains wash out surface soil resulting in adverse effects on inhabitant microflora. Further, avoiding pollution of water bodies such as oceans, river, or lakes can lead to preservation of phytoplanktons, zooplanktons, and other floating microbes. National Biodiversity Authority in India is emphasizing in situ conservation of biological resources including microorganisms. Weinbauer and Rassoulzadegan (2007) highlighted about microbial extinctions

and suggested to conserve microorganisms closely associated with plants and animals that are at risk of extinction, especially inhabiting an endemic environment. A possible example is *Sulfolobus* species, heat-loving microorganisms that inhabit in hot springs. Another motivation for conservation of microorganisms of specific taxa is that they produce potentially important products such as secondary metabolites with pharmaceutical applications and novel enzymes that have higher tolerance capacity against diverse environment. For example, *Thermus aquaticus,* a thermophilic bacterium, produce a heat-resistant polymerase enzyme that is being used in polymerase chain reaction.

Microbial conservation efforts should also lead to the protection of habitat that is responsible for operation of specific function in ecosystem and similar community could be developed to perform certain functions in future. The plant and animal cannot survive in the extreme environmental conditions but microorganisms live happily there. Microbial conservation in Antarctica has been recognized as an important priority to protect biodiversity and their biotechnological potential (Prakash et al. 2013). An example of this category is Endoliths that inhabit the rocks on extreme environments, such as Antarctica. Dry Valleys or deserts around the world may receive special protection (Vincent 2000). Gerrath et al. (2000) suggested the conservation of the rock-dwelling microbial communities of the Niagara Escarpment, Canada. Some microbial ecosystems require specific physical and chemical conditions that need attention for conservation. For example, the protection of microbiota present nearby or in lakes is dramatically affected by changes in water geochemistry (Allgaier et al. 2007).

22.2.2 Ex Situ *Preservation*

Ex situ preservation of microorganisms by individuals and institutes/organizations is a good effort that incorporates the microbial taxa and communities into preservation programme. Proper maintenance and preservation of microorganism's ex situ requires a superior laboratory facility with all the requisite equipments. This strategy includes the gene banks, culture collections, and microbial resources centers making the repository for microbial isolates and keeps away from the time-consuming re-isolation protocols. Conservation of microorganisms through preservation approach is supported by the World Federation for Culture Collection (WFCC) and Directory of Collection of Cultures of Microorganisms. In India, this work is being carried out under the aegis of Ministry of Agriculture & Farmers Welfare, Ministry of Environment, Forests and Climate Change, Department of Science and Technology, Department of Biotechnology and Department of Agricultural Research and Education. The ex situ collections of microorganisms are an essential resource for the future as these are linked to the research programmes and developmental aspects of the country. Regular funding for collection centers and emphasis on education and research will broad the base of microbial diversity research.

22.3 Culture Collections

In general, diverse group of microorganisms are deposited in the culture collection centers to preserve the natural treasure ex situ. Culture collection centers act like a repositories which acquire, authenticate, preserve, catalogue, supply viable cultures of standard reference microorganisms, and provide related services to the research institutes, universities, and colleges. Furthermore, culture collections have crucial role to play as part of patent deposits, providing confidential services for research and industries (Overmann 2015). In most of the cases, microorganisms reported in the scientific papers require prior submission of cultures to a culture collection which revalidates the authenticity of the culture obtained from the researchers (Sharma and Shouche 2014; Smith 2003; Uruburu 2003).

Globally, some of the microbial culture collections acquired the status of International Depository Authority (IDA) for the purpose of patent under the rule of 13.2(a) of the regulations under Budapest Treaty. Currently, this facility is available in 23 countries worldwide at 45 culture collection centers, list and the details of which are available on website of World Federation for Culture Collections (WFCC). In India, only two culture collections have acquired the status of IDA as given below (Sharma and Shouche 2014):

1. Microbial Type Culture Collection and Gene Bank (MTCC), CSIR-Institute of Microbial Technology (IMTECH), Council of Scientific and Industrial Research, Chandigarh, U.T., India
2. Microbial Culture Collection (MCC), CSIR-National Centre for Cell Sciences, Pune, Maharashtra, India

Under the Budapest Treaty, international recognition is given to some culture collections for patent purpose. This treaty is an international treaty signed in Budapest, Hungary, on April 28, 1977. It entered into force on August 9, 1980, and was later amended on September 26, 1980. The treaty is administered by the World Intellectual Property Organization (WIPO). Till 2014, 79 countries were party to the Budapest Treaty. The accession to the Treaty is open to states party to the Paris Convention for the protection of industrial property of 1883. The African Regional Industrial Property Organization (ARIPO), the Eurasian Patent Organization (EAPO), and the European Patent Organisation (EPO) have filed a declaration of acceptance under Article 9(1) (a) of the Treaty.

The treaty allows "deposition of microorganisms at an international depositary authority recognized for the purpose of patent procedure". Usually, in order to meet the legal requirement of sufficiency of disclosure, patent applications and patents must disclose in their description the subject matter of the invention in a manner sufficiently clear and complete to be carried out by the person skilled in the art. When an invention involves a microorganism, completely describing the said invention in the description to enable third parties to carry it out is usually impossible. That is why, in the particular case of inventions involving

microorganisms, a deposit of biological material must be made in a recognized institution. The Budapest Treaty ensures that an applicant, i.e. a person who applies for a patent, need not deposit the biological material in all countries where he/she wants to obtain a patent. The applicant needs only to deposit the biological material at two recognized institutions (one in own country and another outside country), and this deposit will be recognized by all countries party to the Budapest Treaty.

22.4 International Culture Collections

Preserving microbial wealth for future use is the main concern today. The usefulness of potential microorganisms must be exploited in different areas. Culture collections play a very important role in providing microbial resources for the research work and industrial uses. Potential microorganisms are exploited in the industries for the production of vivacious drugs and products for application in agriculture. The Convention on Biological Diversity (CBD) allows sustainable use of potential microorganisms. In 1963, the World Federation for Culture Collections (WFCC) was established with concern for collection, authentication, maintenance, and distribution of cultures and related services, to provide liaison and set up and information network between the collection centers and provide long-term endurance of important collections and pioneer the development of an international database on culture resources worldwide. This data centers is now maintained at National Institute of Genetics (NIG), Japan. WFCC is a culture collection information web portal that manages the information regarding the culture collections registered worldwide and is governed by the World Data Centre for Microorganisms (WDCM). According to this web portal, there are a total of 712 culture collections registered across 73 countries and region with a total number of 2,549,969 (twenty five lakhs forty nine thousand and nine hundred sixty nine) microorganisms. A rank-wise list of countries with their microbial holdings is given in Table 22.1. Some internationally known culture collections are discussed hereunder.

22.4.1 American Type Culture Collection (ATCC), USA

ATCC is an independent, private, non-profit biological resource centers (BRC) and research organization established in 1925. ATCC authenticates microorganisms and cell lines, and manages logistics for long-term preservation and distribution of cultures to the scientific community. As a research organization, ATCC works to generate new knowledge and technology, as well as to continuously improve its function as Biological Resource Centers (BRCs). The scientists of ATCC develop new in vitro model systems, describe new species, search for new disease

Table 22.1 List of countries with total microbial cultures holdings

Rank	Countries and regions	Total holding
1	United States of America	2,61,637
2	Japan	2,54,830
3	India	1,94,174
4	**China**	**1,87,661**
5	Korea	1,14,494
6	Brazil	1,02,066
7	Thailand	99,376
8	Germany	95,593
9	Netherlands	93,421
10	Denmark	88,566

biomarkers and build bodies of characterization data for valuable biological materials. In addition, they study improved methods for characterization, long-term preservation and proper authentication of biological materials.

22.4.2 Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ), Germany

Leibniz Institute DSMZ–German Collection of Microorganisms and Cell Culture is one of the largest bioresources centers worldwide. Its collections currently comprise almost 40,000 items, including about 20,000 different bacterial and 5000 fungal strains; 700 human and animal cell lines; 800 plant cell lines; 1000 plant viruses and antisera; and 4800 different types of bacterial genomic DNA. All biological materials accepted in the DSMZ collection are subject to extensive quality control and physiological and molecular characterization. In addition, DSMZ provides an extensive documentation and detailed diagnostic information on the biological materials. The unprecedented diversity and quality management of its bioresources render the DSMZ an internationally renowned supplier for science, diagnostic laboratories, national reference centres, as well as industrial partners.

The trans-sectorial research of the DSMZ focuses on (1) microbial diversity and the underlying evolutionary mechanisms (genome evolution, population genetics), (2) improved methods for the access and ex situ preservation of biodiversity, as well as (3) molecular mechanisms of biological interactions (symbioses, mechanisms of disease, cancer). DSMZ maintains specific expertise and offers counselling in the areas: (1) microbial taxonomy, phylogeny and species description, (2) standardization and quality assurance of bioresources, (3) biosafety and biosecurity, and (4) legal framework for the exploitation of bioresources (patenting, convention on biological diversity, access and benefit sharing).

22.4.3 Commonwealth Agricultural Bureau International (CABI), UK

The CABI is an international not-for-profit organization that improves people's lives by providing information and applying scientific expertise to solve problems in agriculture and the environment and was established in 1910. Besides knowledge sharing and science, CABI helps in addressing issues of global concern such as improving global food security and safeguarding the environment. They do this by helping farmers grow more and lose less of what they produce, combating threats to agriculture and the environment from pests and diseases, protecting biodiversity from invasive species, and improving access to agricultural and environmental scientific knowledge. The CABI has 48 member countries guide and its areas of work are development and research projects, scientific publishing and microbial services. Its registered office is situated in Egham, England. CABI housed a collection of over 28,000 living strains including the UK National Collection of Fungus Cultures. The culture collection contains over 6000 species isolated from environmental and agricultural systems worldwide. It supplies authenticated reference, type and test strains to businesses and research establishments. CABI maintains ISO846, BS2011 Part II j and Mil Std 810G test strains from the European, the UK and US testing standards, and holds many other test and challenge strains.

22.4.4 China Centre for Type Culture Collection (CCTCC), China

China Type Culture Collection (China Centre for Type Culture Collection, referred CCTCC) was established in 1985 by the State Intellectual Property Office (formerly the Chinese Patent Office). The CCTCC deposits microorganisms including bacteria, actinomycetes, yeasts, fungi, unicellular algae, human and animal cell lines, genetically modified cells, hybridomas, protozoa, lichens, plant tissue culture, plant seeds, animal and plant viruses, phages, plasmids, and gene libraries and other types of microorganisms (biomaterials/species). The CCTCC union got International Depository Authority, IDA recognition on July 1, 1995. It preserves all kinds of cultures from 22 countries, where more than 3800 are patent culture strains, more than 15,000 strains of generic culture, 1000 culture of microbial type strains, more than 1000 strains of animal cell lines, and more than 300 strains of plant and animal viruses.

22.5 Indian Culture Collections

India is one of the hotspots of biodiversity in the world owing to its varied climatic conditions and has been divided into 22 agrobiodiversity hotspots. The convention on biological diversity was signed at Rio in 1992. Microbial diversity analysis is a dynamic area of research as it provides vast data which can be useful in agriculture and industry. At present, only fraction of microbial life (less than 5 %) is known and explored. Richness of microbial diversity is one of the reasons why we are at second position in the total number of holding of microbial cultures. In India, there are 29 culture collections registered with the WFCC, but not all are functional now and few collections are preserving only a few microorganisms. The major culture collections recognized as Designated Repositories (DRs) by National Biodiversity Authority (NBA) and Ministry of Environment, Forests & Climate Change for microorganisms are as follows:

22.5.1 Microbial Type Culture Collection and Gene Bank (MTCC), CSIR-IMTECH, Chandigarh

The Microbial Type Culture Collection and Gene Bank (MTCC), a national facility established in 1986, is funded jointly by the Council of Scientific and Industrial Research (CSIR) & Department of Biotechnology (DBT), Government of India. It is well equipped with the modern facilities and is housed in the Institute of Microbial Technology (IMTECH), Chandigarh. It is an affiliate member of the World Federation for Culture Collections (WFCC) and is registered with the World Data Centre for Microorganisms (WDCM). The main objectives of this national facility are to act as a depository, to supply authentic microbial cultures, and to provide related services to the scientists working in research institutions, universities, and industries. MTCC was recognized by the World Intellectual Property Organization (WIPO), Geneva, Switzerland, as an International Depository Authority (IDA) on 4 October, 2002, thus becoming the first IDA in India, seventh in Asia, and thirty-fourth in the World. The deposit of microorganisms under the Budapest Treaty is recognized to fulfil the requirement of patent procedure in 55 member countries. Presently, MTCC has five sections, Actinomycetes, Bacteria, Fungi, Yeasts, and Plasmids and holds over nine thousand cultures.

22.5.2 Microbial Culture Collection (MCC), DBT-NCCS, Pune

Microbial Culture Collection (MCC) is located at National Centre for Cell Science, Pune. The MCC was established by the Department of Biotechnology (DBT), Government of India, in 2007. In April 2011, MCC was recognized by the WIPO, Geneva, Switzerland, as an International Depository Authority (IDA) for the deposit of patent

microorganisms under the Budapest Treaty and also as a Designated National Repository by the Ministry of Environment, Forests and Climate Change. MCC holds the largest collection of microorganisms in India and second largest in the world but all of them are not characterized. The main objective of this collection center is isolation, identification, preservation of microorganisms from various niches, development of new strategies for isolation of "uncultivable" microorganisms, and providing consultation services for patent deposits, preservation, propagation, biodeterioration, industrial problems, biosystematics, and microbial biodiversity issues, etc.

22.5.3 National Agriculturally Important Microbial Culture Collection (NAIMCC), ICAR-NBAIM, Maunath Bhanjan

Indian Council of Agricultural Research (ICAR) has established ICAR-National Bureau of Agriculturally Important Microorganisms (ICAR-NBAIM) at old building of National Bureau of Plant Genetic Resources, New Delhi with aim for exploration, collection, maintenance, preservation and supply of microorganisms of authentic microbial cultures to researchers, farmers and industry Subsequently, the Bureau was shifted to present location, Maunath Bhanjan, Uttar Pradesh, on 1st June 2004. National Agriculturally Important Microbial Culture Collection (NAIMCC) was established in same year as a Unit of ICAR-NBAIM. The objectives of NAIMCC are to serve as National Repository to conserve agriculturally important microbial germplasm and to provide reliable microbial cultures along with other services to teaching and research institutions, farmers, and industry. The NAIMCC is working in accordance with the guidelines of the World Federation for Culture Collections (WFCC). It has been an affiliate member of WFCC since 2014 with World Data Centre for Microorganisms (WDCM) with Reg. No 1060. The NAIMCC has microbial holdings of more than 6000 AIMs including bacteria and actinomycetes (2202), fungi (3690), and cyanobacteria (152).

22.5.4 Indian Type Culture Collection (ITCC), ICAR-IARI, New Delhi

A national herbarium established by Sir. Edwin John Butler at Pusa, Bihar, in 1905 was later shifted to Indian Agricultural Research Institute in 1934. The Herbarium Cryptogamae Indiae Orientalis (HCIO) is the oldest fungal herbarium in India for preserving the fungal biodiversity in the form of specimens. The Indian Type Culture Collection (ITCC) was established in 1936 with a view to conserve, catalogue, identify, and furnish the knowledge on living fungi and bacteria. It is the oldest fungal genetic resource centre in India. More than 50,000 fungal disease

specimens at HCIO and 3900 microbial strains/isolates at ITCC which includes plant pathogens, biocontrol agents, fungi for medical and industrial use including mushrooms and yeasts from various ecological niches like soil, plant, forests, and insects, etc., are being conserved and maintained. The main objective of HCIO and ITCC is to act as repositories for diseased specimens, and fungal and bacterial cultures, respectively, and to provide services, *viz*., supply and identification of fungal and bacterial cultures to various technocrats and scientists working in research institutions, universities, and commercial organizations.

22.5.5 Centre for Conservation and Utilisation of Blue Green Algae (CCUBGA), ICAR-IARI, New Delhi

The Center for Conservation and Utilization of Blue Green Algae (CCUBGA) is established in Microbiology division of IARI, New Delhi. The CCUBGA within the Division are the major provider and storehouse of the national microbial wealth. The number of cyanobacterial strains in the collection center is more than 550. These represent a collection of a large number of genera and species isolated from a wide variety of habitats. Various cyanobacterial strains of same species but varying in their colour and area of isolation are available. To differentiate them, center has assigned a traditional genus and species name under the Botanical Code System to each strain followed by a Cyanobacterial Culture Collection (CCC) number on the basis of period of their isolation and deposit at the center.

22.6 Other Indian Culture Collections

22.6.1 National Fungal Culture Centre of India (NFCCI), DST-ARI, Pune

In an initiative of Department of Science and Technology (DST), Government of India has set up a National Fungal Culture Collection of India (NFCCI) in the campus of Agharkar Research Institute (ARI, Pune) in 2008. NFCCI's core activities are acquisition, verification, preservation and maintenance, morphological and genetic authentication, distribution of identified cultures, and associated information and documentation. As core activities, after verification of purity and taxonomic identity, fungal strains are deposited in NFCCI. Currently, NFCCI holds 4000 live, pure, and morphologically identified indigenous cultures. NFCCI is an affiliate member of World Federation for Culture Collections and is registered with the World Data Centre for Microorganisms (WDCM-932). Its services are presently extended mainly to Indian users in order to satisfy the Indian regulatory compulsions, especially National Biodiversity Act 2002.

22.6.2 National Collection of Industrial Microorganisms (CSIR-NCIM), Pune

National Collection of Industrial Microorganisms (NCIM), a national facility and microbial culture repository situated housed in National Chemical Laboratory (NCL), Pune, is dedicated to isolation, preservation, and distribution of authentic and industrially important microbial strains. Established in 1951, the NCIM is one of the oldest biological resource centres holding especially industrially important microorganisms in the country. Collection holding currently comprises almost 5000 items, including bacteria, fungi, actinomycetes, yeasts, and algae. These cultures produce industrially important metabolites such as enzymes, antibiotics, organic acids, pigments, etc.

22.6.3 Veterinary Type Culture Collection (VTCC), ICAR-NRCE, Hisar

Veterinary Type Culture Collection (VTCC) at National Research Centre on Equines, Hisar, holds microorganisms of veterinary and dairy importance. VTCC is the first facility in livestock sector in India for collection of animal microbes comprising veterinary, rumen, and dairy microbes. The activities include isolation, characterization, conservation, maintenance, and distribution of these microorganisms including phages for their utilization in animal health and production. So far, a total of 2546 cultures/clones have been deposited in the VTCC repository after authentication and conventional and molecular characterization including GC-FAME and sequence analysis of 16S rRNA and other virulence genes. These microbes have been isolated as well as received as deposits from 19 network units, including veterinary (7), rumen (8), and dairy (4) network units, and other ICAR institutes and State Agricultural and Veterinary universities. These cultures/clones include veterinary pathogens including viruses, bacteria, bacteriophages, clones, rumen microbes comprising anaerobic bacteria, fungi, and methanogenic archaea and dairy microbes. The overall benefit of VTCC activities would result in enhancement of knowledge in all the spheres of animal microorganisms including utilization of their useful properties.

22.6.4 Veterinary Type Cultures of Microorganisms (VTCM), ICAR-IVRI, Izatnagar

Veterinary type culture laboratory was initiated at IVRI in the Division of Biological Standardization during 1970. The main objective of the laboratory is to maintain, preserve, and supply standard bacterial, viral, fungal, and parasitic cultures used in production and quality control of veterinary biologicals for R&D and for teaching. At IVRI, more than 488 bacterial and viral cultures are being maintained in different laboratories. The Division of Biological Standardization functions as nodal agency for

veterinary type cultures and coordinates maintenance and supply of standard microbial cultures with other specialized laboratories of different Divisions. VTCM focuses on harmonizing production, standardization, and quality control of veterinary biologicals in India and neighbouring countries by acting as a nodal agency for the supply of vaccine and challenge strains of bacteria and viruses.

22.6.5 Mushroom Culture Collection, ICAR-DMR, Solan

The importance of mushroom culture collection was visualized with the establishment of ICAR-Directorate of Mushroom Research, Solan (earlier NRCM) in 1983. It was just a small beginning with few mushroom cultures obtained from Research Scientists of Solan and Plant Pathology Division, IARI, New Delhi, and these cultures were maintained for the research work of Scientists of ICAR-DMR, Solan. Today, it is one of the largest mushrooms Gene Bank in India preserving the valuable mushroom germplasm and supplying mushroom cultures to researchers and mushroom entrepreneurs. Today, gene bank of ICAR-DMR is having 2783 accessions of mushroom cultures, namely, *Agaricus* spp., *Pleurotus* spp., other edible fungi, and cultures of wood-rotting, mycorrhizic, and poisonous fungi.

22.6.6 Repository for Cyanobacteria and Microgreen Algae, DBT-IBSD, Imphal

National repository for cyanobacteria and microgreen algae (Freshwater) is a national facility created by the Department of Biotechnology (DBT), Govt. of India at IBSD, Imphal, Manipur, a North-Eastern State of India. The North-Eastern region (NE region) of India (located between 87°32′E to 97°52′E longitudes and 21° 34′N to 29°50′N latitudes) including Indo-Burma hotspot is 8th among the 34 biodiversity hotspots of the world and is one of the two biodiversity hotspots in India. This region has a wide range of physiographic and eco-climatic conditions which supports different forms of life. Collection of the samples has been done from different ecological niches of North-Eastern region of India. The repository has one thousand seven hundred and six (1706) uni-algal cultures, out of which one thousand two hundred and forty nine (1249) are cyanobacterial cultures and four hundred and fifty seven (457) are microgreen algae. Five hundred eight (508) uni-algal cyanobacterial strains have been screened for fine chemical and secondary metabolites, namely, chl-a, total soluble proteins, total carbohydrates, ammonia excretion, carotenoids, and phycobili proteins. Two hundred one (201) uni-algal heterocystous cyanobacterial strains have been investigated for the estimation of nitrogenase activity by acetylene reduction activity (ARA). Eighty-two (82) cyanobacterial strains have been subject to lipid profiling and fatty acid composition by using GC-FID methods. One hundred and thirty seven (137) cyanobacterial strains have been characterized at molecular level and allotted NCBI accession number.

22.6.7 Microbial Culture Collection, Indian Spice Research Institute, Kozhikode

ICAR-Indian Institute of Spices Research as a research entity has key interest in spice crops, especially black pepper, cardamom, ginger, turmeric, nutmeg, clove, cinnamon, all spices, and vanilla. All these spice crops are highly vulnerable to fungal, bacterial, and viral pathogens resulting in huge economic losses to the farmers in the country and elsewhere. Diseases like *Phytophthora* foot rot, slow decline, anthracnose in black pepper, capsule rot, rhizome rot, leaf blight in small cardamom, leaf spot in ginger and turmeric, bacterial wilt in ginger, etc., have taken a heavy toll on the crops. Considering the importance, ICAR-IISR maintains culture collections of major pathogens of important spice crops, potential endophytic as well as rhizospheric antagonistic microbes, and plant growth-promoting rhizobacteria, among which few are commercialized.

22.6.8 Microbial Resources at ICAR-IIHR, Bengaluru

Microbial resources at ICAR-IIHR, Bengaluru, are held in by individual scientists working on various aspects of horticulture spread over five divisions. The Division of Soil Science and Agricultural Chemistry, at ICAR-IIHR is small repository/collection of agriculturally important microbes of plant and soil origin. The well-characterized resources of this division have been deposited at NAIMCC and various national repositories. The Division of Plant Pathology established a culture collection with holdings of more than 500 fungal pathogens namely of important fungal pathogens, *viz., Alternaria* spp., *Fusarium*, *Phytophthora*, *Colletotrichum*, and *Ceratocystis*. The division has also deposited bacterial pathogens and biological control agents at NAIMCC. The division also maintains around 90 mushroom germplasm. The Division of Entomology and Nematology has nematicidal fungi and bacteria which have been deposited at NAIMCC and ITCC. The Division of Postharvest Technology holds a collection of yeast and lactic acid bacterial strains. The Division of Biotechnology has a collection of endophytic and *Bacillus thuringiensis* strains, which have been accessioned at NAIMCC.

22.6.9 National Culture Collection of Pathogenic Fungi (NCCPF), ICMR-PGIMER, Chandigarh

The National Culture Collection of Pathogenic Fungi (NCCPF) is the national facility established in PGIMER, Chandigarh, during 2010, sponsored by the Indian Council of Medical Research (ICMR), New Delhi, for the deposition, maintenance, identification, and supply of all pathogenic fungi. NCCPF have maintained over 2500 strains of fungi, both filamentous and yeasts, representing a large percentage of the pathogenic

species in the fungal kingdom. The main aim of the center is to set up a facility of international standard for preservation and to handle deposits of medically important fungi and to supply authentic strains to investigators in India.

22.6.10 National Collection of Dairy Cultures (NCDC), ICAR-NDRI, Karnal

The National Collection of Dairy Cultures (NCDC) has been established in the Division of Dairy Microbiology, National Dairy Research Institute, Karnal. The cultures were initially maintained on solid media by periodic subculturing. However, freeze-drying was introduced in 1964 for long-term preservation of cultures. A total of 420 cultures were held at NCDC to date. The NCDC stock includes strains of bacteria, yeasts, and moulds of general interest to education, research, and institution. The main interest of the collection lies in the lactic acid bacteria from dairy sources. In addition to individual strain cultures, the NCDC also holds mixed strains (traditional) and defined strain-formulated cultures for making different varieties of cheese and fermented milk products. The NCDC also organizes specialized training programmes and workshops to provide know-how on the maintenance and preservation of cultures, propagation of dairy starters, and other related aspects.

22.6.11 Goa University Fungus Culture Collection and Research Unit (GUFCC), Goa

Goa University Fungus Culture Collection and Research Unit has been established in 1999. The collection center acts as an ex situ repository of ecologically and economically important fungi besides terrestrial actinobacteria in the state of Goa. The aim of the centre is to develop and modify existing protocols for culture isolation, screening, etc., create photo and video documentation of fungal resources for global use, and aid national culture collections by depositing representative fungi useful for national research and development. It also provides educational and research opportunities to PG and doctoral research students, and service academic and commercial clients with high-quality fungal cultures for use in educational, research, and industrial applications.

22.6.12 National Facility for Marine Cyanobacteria (DBT-BDU), Tiruchirapalli

Considering the biotechnological potentials of marine microbes, National Facility for Marine Cyanobacteria sponsored by DBT, India, has been established at

Bharathidasan University. It is the only facility in the world dedicated solely to the field marine cyanobacterial research. The main focus of the center is to survey the entire coastline of India and establish germplasm collection of marine cyanobacteria as well as cryophilic cyanobacteria.

22.7 Agriculturally Important Culture Collections of the World

Microorganisms are utilized to develop biofertilizer and biopesticide to enhance the plant growth and development by inhibiting the growth of weeds and insect pests and control the disease of plants. Microorganisms also allow plant to absorb more nutrients from the soil and recycle the complex by converting into the simpler form to be utilized by plant. In return, plant provides its waste material to be utilized by microorganisms. Several culture collections have been established worldwide after realizing the importance and potential of microorganisms in agriculture (Table 22.2). Some specialized groups of microbial culture collections have been also established (Table 22.3).

Table 22.2 Agriculturally important culture collections in Asia and other parts of the world

Culture collection	Country
Agriculture Culture Collection of China (ACCC)	China
Korean Agricultural Culture Collection (KACC)	Korea
National Collection of Agricultural and Industrial Microorganisms	Hungary
Culture Collection Beijing Agricultural University (CCBAU)	China
International Collection of Microorganisms from Plants	New Zealand
Russian Collection of Agricultural Microorganisms (RCAM)	Russian Federation
United States Department of Agriculture (USDA)	USA

Table 22.3 Some specialized microbial culture collections

Culture collection	Country
Swiss Collection of Arbuscular Mycorrhizal Fungi (SAF)	Switzerland
International Culture Collection of Arbuscular Mycorrhizal Fungi (INVAM)	USA
Industrial Yeast Collection	Italy
Collection for Extremophile Microorganisms and Type Cultures (EMTC)	Russian Federation
Marine Culture Collection of China	China
USDA-ARS Collection of Entomopathogenic Fungal Cultures	USA
USDA-ARS National Rhizobium Germplasm Collection	USA

22.8 Microbial Culture Techniques

Cultivation of microorganisms is a method applied to grow and multiply microbial cells under the predefined chemical medium and controlled environment. Although the cell cannot be seen with unaided eye until it forms a colony. A colony is a group of cells all originated from a single cell or cluster of cells of similar or different kinds of cells. All individual organisms in the colony are of the same species. Microbial cultures help to study and research more about these microorganisms, their characteristics, and identity. This information helps in determining whether they are beneficial or harmful to us. To grow and study these microorganisms, one need to take care of their food and environmental conditions.

Like many living systems, microorganisms also require energy to perform its metabolic functions. These energy sources range from carbon, nitrogen, oxygen, iron, and other minerals, micronutrients, and water. All these nutrients are essential for the growth and multiplication of cell. The medium which we use to grow these microorganisms may be synthetic, semi-synthetic, or completely natural.

a. Synthetic medium: components of the medium which are not of the natural origin, i.e. containing salts and minerals, are called synthetic medium.
b. Semi-synthetic medium: medium which is a combo of both natural and chemical component are called semi-synthetic medium.
c. Natural medium: components in this medium are of natural origin.

The carbon source added in the medium provides energy and carbon skeleton for synthesis of various other carbon compounds. In addition to the chemical added to the medium, growth factors are also supplemented in the medium which are essential for the growth and multiplication of the cells. Water is added as the base of the medium whether it is liquid or solid. The liquid medium contains the nutrients dissolved in the water; this is also often referred as broth culturing. In broth medium if you add the desired quantity of agar, it forms agar medium and it also enables the medium to get solidified. Culturing of microorganisms in broth shows diffused growth and helps in studying growth phase of microorganisms and for bioprospection studies. Whereas the solid medium helps in isolating pure culture which forms distinct colony on agar medium. It is also easy to study the culture characteristics, morphological diversity and biocontrol assays on solid medium but it is not possible in the broth culture.

Microorganisms are prevalent in diverse niches ranging from hot to cold environments. Some are acid loving, some like moisture, and some can tolerate high saline conditions. Many require the presence of oxygen (aerobic), while others do not require (anaerobic) oxygen. Environment has a great impact on the growth and multiplication of microorganisms. The factors which affect metabolic state of microorganisms are ionic strength, pH, and temperature. Most of the bacteria grow at neutral pH, whereas yeast and fungi require slightly acidic pH. Similarly, different microorganisms require different optimum temperature for the active growth and multiplications. Microorganisms which show maximum growth at

temperature 0–20 °C are called psychrophiles, those at temperature 25–40 °C are called mesophiles, and those at temperature with temperature 45–122 °C are called thermophiles. In routine manner, the optimum conditions are maintained in the laboratory with the help of an incubator.

22.9 Method of Maintenance

Maintenance comes into play once the culture is pure. The pure culture thus obtained has a chance of contamination from the other dominant microorganisms present in the environment. So, to protect the isolated, pure culture of microorganisms from contamination and to initiate research on organisms, one generally prepares sufficient numbers of stock cultures and working cultures.

Stock culture is prepared by inoculating the pure culture of a bacterium to fresh medium and making multiple copies of it. Then the plate is kept in the incubator to obtain optimum growth. After this, each plate is sealed using a parafilm tape and placed in the refrigerator, i.e. at 4 or 20 °C. Care is taken to place each set at different location to ensure that the culture is not totally lost. If a refrigerator at one place in not working and the cultures stored in that refrigerator are lost, the culture stored in the other refrigerator can be revived.

Stock cultures are often maintained on the agar plates, agar slants, and in the form of stab cultures. Broth culture is not preferred at all for this purpose. Under normal conditions, the microbial culture utilizes the nutrients present in the medium and excretes toxic material in the medium. This toxic material thus alters the pH and the gaseous phase of the medium and makes the environment not so suitable for the culture itself and shows decline in the growth. This leads to the necessity of subculturing wherein the microbial culture is transferred to a fresh medium. To escape from frequent subculturing, the microbial cultures are often stored at low temperature. As we know that the growth and the metabolism of the microorganism slow down at low temperature which the refrigerator maintains, but this does not totally halt the metabolic activity of the microorganisms. As a result, the medium provided is slowly consumed by them and then slowly a stage will be reached when the medium will not be sufficient to feed all microorganisms. This induced stress may lead to an alteration in the genome of the microorganisms which is definitely not preferred. Therefore, periodic observation should be made after specific time interval and if need be the organism is subcultured again.

Subculturing of pure organisms is being carried out on fresh medium, i.e. on Agar medium in Petri dishes and slants. Here multiple copies are prepared and stored in refrigerator. As per the requirement, one of the culture plates is opened and used to perform different assays. Periodic monitoring is also required here to check for contamination. Once number of the culture reduces to one or two, more new subcultured plates are added to maintain sufficient amount of culture. In case of loss of cultures and change in phenotypic characteristics due to environmental extremity, the stock culture may be used to replenish the existing stocks. Although this

method has a lot of disadvantages, but it is still followed in most of the laboratories for all kinds of microbial cultures, i.e. bacteria, fungi, yeast, etc. In general, the medium which promotes good sporulation is considered to be the most desirable (Gherna 2009). It is always better to cultivate cultures on slant medium as it occupies small space and there is little chance of contamination as it is very well covered by the cotton plug. Transfer should always be made from the growing end of the colony. Some fungi degenerate when maintained on the same medium for the extended period of time. Thus, the medium should be altered time to time (Gherna 2009). The time interval to subculturing of fungus varied with respect to the fungal culture. It generally requires 2–4 weeks, but certain cultures may survive for 12 months without subculturing. The maintenance of fungal cultures by serial transfer is a challenge as there is always a chance of contaminated variants getting selected, mislabelling of cultures, infestation with mites, and labour intensity.

Microbial cultures of yeast can be easily maintained by serial transfer on either solid or liquid medium. Yeast survives for quite a long time on solid agar medium as compared to the liquid broth medium, especially non-fermentative yeast. A series of changes further accumulate and might result in completely different phenotypic character as acquired. This method is very simple and inexpensive which make it suitable to be followed in most of the laboratories. To reduce frequent subculturing, one also prefers to store it in refrigerator. Cooling helps in decreasing the growth rate leading to decreased frequency of subculturing.

22.10 Method of Preservation

Preservation of microbial culture includes all bacteria, fungi, algae, archaea, virus, protozoans, and yeast. For publishing research paper on novel strain of bacteria, it is mandatory to submit the same microbial culture at two recognized culture collection centres to ensure its preservation. In other words, it is a voucher to achieve publication. Before proceeding to preservation, there are many points which need to be addressed; these include the type and structure of microbes to be preserved, the intended use of the material, and the facilities and expertise available. There are many preservation techniques which can be used for long-term and stable storage. Freeze-drying and liquid drying often apply to bacteria and can be successful for some of the other types of microorganisms, but techniques fail to work for most vegetative states (Smith et al. 2008). Cryopreservation plays a very important role in the long-term preservation of microorganisms.

The preservation techniques may be classified into three types based on the duration for which the culture can be stored and preserved.

- Short-term preservation
- Medium-term preservation
- Long-term preservation

22.10.1 Short-Term Preservation

22.10.1.1 Refrigeration

This method is still the most preferred method for preservation of microbial cultures for short duration at 4 °C. In refrigerator, cultures can be stored which are used daily or weekly and cultures grown on agar slants or plates with precautions to avoid contamination. Active cultures should be sealed with parafilm before storing. Sealing of petri dishes protects the plates from contamination and drying of agar. For slants, screw-capped tubes are used. In this method, vials are filled with agar medium and then sterilized. Thereafter, microorganism is inoculated aseptically. Cultures are then stored with loose cap overnight and then stored with tight caps at 4 °C. The cultures are periodically transferred to fresh medium to retain viability.

22.10.2 Medium-Term Preservation

22.10.2.1 Mineral Oil

Storage in mineral oil or paraffin oil is one of the earlier methods used for the preservation of fungal cultures for extending the longevity of culture. It is cheap and easy method of preservation for quite a long time and is recommended for laboratory with limited resources. Cultures can be stored for several years (10–12 years) without undergoing much physiological change at room temperature (25–30 °C. In exceptional case, a culture can be stored for 32 years at room temperature of 15–20 °C (Fig. 22.1). This method is most suitable for mycelial or

Fig. 22.1 Preservation and storage of fungi in mineral oil

non-sporulating cultures which do not withstand freezing or freeze-drying. The principle behind this type of preservation is that mineral oil/paraffin oil prevents evaporation from the cultures and decreases the metabolic rate of the culture by limiting the oxygen supply. Culturing with this method also protects from contamination of fungi and mite infections (Homolka 2014). The disadvantage of this method is that it doesn't completely stop the growth of the fungi and the one which grows may be a mutant. This method is suitable for the preservation of mushroom fungi (mainly wood-rotting basidomycete) but not for Zygomycotina (Homolka 2014).

22.10.2.2 Silica Gel

Silica gel is used for the preservation of spore-forming fungal cultures. In this method, spore suspension and the silica gel are mixed and stored for quite a long duration. Anhydrous silica gel (6-22 mesh, non-indicator) is dried in an oven at 180 °C for 3 h by filling it to the quarter of the cultural tube. After heating, it is allowed to cool and mixed with ethylene glycol and dry ice. Care must be taken to ensure uniform cooling. Another alternative is to keep it in freezer at a temperature range of −15 to −20 °C. Prepare fungal spore suspension on the other side by suspending spores in chilled 5 % skim milk solution. It is further added to the chilled silica gel. Avoid saturation of the gel by filling the culture tube not beyond three-quarters. The culture tube is kept for ~20–30 min with intermittent agitation which ensures uniform dispersion. The cultures are held at 25 °C for ~1–2 weeks to separate out crystals when shaken. Inoculated bottles are sealed with screw cap and stored on an indicator silica gel. To revive culture of the fungal spores, inoculate on appropriate medium and incubate at suitable temperature. It has shown good results with yeast showing viability after 2–5 years (Gherna 2009; Smith et al. 1984). In a method suggested by Perkins (1962), a stock culture of *Neurospora crassa* in anhydrous silica gel has proved consistently useful and effective in preservation.

22.10.2.3 Soil

Fungi can be easily maintained on soil. This method is very simple and uses the nutrients in the sterile soil (free of living microorganisms) as the source of food. Before inoculating the spore suspension of fungi, soil is autoclaved twice at 121 °C and 15 lbs pressure for 15 min duration. After sterilization, the spore suspension is properly mixed in the soil maintaining the sterile environment. The mixture thus obtained is further incubated for 5–10 days and then stored in the refrigerator at 4 °C. In this manner, the fungi can be preserved for longer duration of 2–5 years. Revival of fungi can be done by providing the appropriate medium and followed by incubation.

22.10.2.4 Sterile Water

Preservation of microorganisms in sterile distilled water was first given by Castellani and, therefore, is often referred as Castellani method (Castellani 1963) which is applied on *Entomophorales, Pyrenomycetes, Hymenomycetes, Gasteromycetes*, and *Hypomycetes*. It was found to apparently suppress the morphological change in many fungi. Basidomycetes were successfully preserved for 2 years at 5 °C (Ellis 1979; Marx and Daniel 1976; Richter and Bruhn 1989), although viability decreased after 5–10 years of storage (Burdsall Jr and Dorworth 1994). Preservation of fungi in distilled water is a cheap and practical method to maintain a mycology collection for a short time interval (Diogo et al. 2005). It has also proved that plant and human pathogenic bacteria can be preserved in pure water or PBS for several years (Liao and Shollenberger 2003). The disadvantage of this method is subculturing in a time interval. It is suitable for the storage of basidiomycetes decay fungi for longer period (Richter et al. 2010). In some cases, the fungi have also been reported to have undergone morphological change alterations during storage and exhibited significant degree of pleomorphism upon revival.

22.10.2.5 Paper Disc for Yeast

Yeast cells are used to be preserved on the filter paper disc. The Whatman filter paper no. 4 is used which is first cut into the size which is required by the user and is sterilized. It is piled and wrapped inside the aluminium foil and sterilized by autoclaving at 121 °C, 15 lbs pressure for 15 min. These sterile discs are inoculated by dipping in the yeast suspension in 5 % skimmed milk for 1 min. The discs are dried in desiccator for 2–3 weeks at 4 °C and then packed in the airtight container and stored at 4 °C. Following this way, cultures can be stored for 2–3 years, and whenever required, a piece of the paper can be inoculated on the fresh medium (Kulkarni and Chitte 2015).

22.10.2.6 L-Drying

Liquid state drying is usually referred to as L-Drying, wherein the sample is protected from freezing. Drying is performed in the vacuum at temperature below 4 °C. Another simple method is given by Malik (1990) in which activated charcoal has been used as the carrier for long-time preservation. In another study of Tommerup (1988), the viability of spores and hyphae of seven species of vesicular arbuscular (VA) mycorrhizal fungi was maintained for 8 years by maintaining low level of vacuum. Sakane et al. (1992) have also worked to preserve extremely halophilic archaebacteria and thermo-acidophilic archaebacteria that are sensitive to freezing and freeze-drying. Drying was performed at 5 °C which increased the survival values for as many as 15 years.

22.10.3 Long-Term Preservation

Long-time freezing is a much preferred technique to preserve microbial cultures for quite a long period. In this technique, media, manpower, and regular subculturing are not required to maintain microbial cultures. Only thing required is the facility which supports microbial cultures at such low temperature. Followings are some specific methodologies involving freezing.

22.10.3.1 Ultralow Freezing (Ultralow freezers)

Ultralow freezing is done by freezers having two compressors wherein each compressor has a different refrigerant which maintains temperature around −80 °C. Cells stored at this temperature can remain viable for several years (Fig. 22.2). This low temperature reduces chemical reactions within the culture. Here, molecular motion still works in frozen cells, and thus, viability of culture will decline. During this type of storage, ice crystals are formed. This ice can damage the cells by dehydration caused by localized increase in salt concentration. This problem can be overcome by the use of cryoprotectants.

Fig. 22.2 Preservation of microbial culture in deep freezing

Fig. 22.3 Cryotanks for preservation of microbes at −196 °C

22.10.3.2 Cryogenic Freezing (Cryogenic Freezers)

In this type of freezing, freezers rely on liquid nitrogen systems to operate. It is known as cryopreservation. It should be less than −130 °C, generally up to −196 °C (Fig. 22.3). At this temperature, the molecular motion of water does not take place and cells are trapped in glass like matrix. Microorganisms stored in cryogenic freezers retain their viability for many years. For microorganisms which are prone to cryogenic injury that exhibit poor viability following preservation, specific protocols are designed to ensure optimal cryopreservation (Smith and Ryan 2012). Decreasing the temperature of microbial cells reduces the rate of metabolism until all internal water is frozen and biochemical reactions do not occur and the metabolism is restricted.

In this method, tubes should never be stored in tanks submersed in liquid nitrogen because screw cap tubes leak and pull the nitrogen into the tube along with contaminants. But liquid nitrogen vapour phase freezers avoid this problem with having disadvantage of being expensive and require large volumes of liquid nitrogen. In general, intracellular freezing is lethal. During this process, intracellular and extracellular waters of the cell change into ice and thus arises the problem of injury. To overcome this problem, cryoprotectants are used with the cells. Although a good survival of deep-frozen microbes (bacteria and microbial spores) has occasionally been observed without a cryoprotectant, the presence of a suitable cryoprotectant usually increases the survival considerably. The agent may provide protection by being intracellular or extracellular (Pichugin 1993).

On the basis of penetration capacity, cryoprotectants are of three different types:

- Cryoprotectants penetrating both cell wall and cytoplasmic membrane, i.e. DMSO and glycerol.
- Cryoprotectants penetrating cell wall but not cytoplasmic membrane, i.e. amino acids, PEG-1000, etc.
- Cryoprotectants not penetrating even cell wall, i.e. high molecular weight proteins, PEG-6000, etc.

The exposure of cells to a high concentration of cryoprotectant causes osmotic dehydration. As the permeating cryoprotectant is removed by exposing the cells to a lower concentration of the compound, the osmotic uptake of water causes the cells to swell above their initial volume. And cells shrink as the cryoprotectant moves out. It returns to physiological volume only if non-permeating solute has neither been lost nor gained during the process. Cells are generally more sensitive to swelling than to shrinkage; thus, removal of cryoprotectants tends to be more hazardous than their addition.

The rate of change of temperature also influences the rate at which water is transported out of the cells during cooling and into the cells during warming. If the cooling rate is rapid for the membrane of the cell in question to transport sufficient water out of the cell, then protoplasm will become supercooled, and the greater the extent of supercooling, the more likely is the chance of the cell freezing internally. The combination of solution effects and intracellular freezing causes each cell to show maximal survival at a characteristic cooling rate; as the cooling rate increases from very low rates, so does survival because the deleterious effects of exposure to high salt concentrations are reduced, but eventually survival drops because intracellular freezing appears.

Freezing rate also affects the process of cryogenic preservation. Presently, it is known that fast freezing is a process which results in the formation of a large number of small-size crystals in the suspension. It is characterized by increase in viscosity. In this type of case, cells can reach to the temperature of −40 °C without changes in their content. As the efficient crystalline nuclei do not form, the supercooling causes homogeneous crystallization in the whole volume without cell membrane damage, whereas slow freezing is characterized by extracellular formation of crystals and cell reaches osmotic equilibrium with the environment by dehydration. These result in two effects: concentration of the cell content and decrease in cell volume. Slow freezing causes over dehydration and contraction of the cell volume below the critical value. In rapid freezing, large numbers of small ice crystals are formed. These small ice crystals produce less cell wall rupture than slow freezing which produces only a few large ice crystals. Here, it is notable that dehydration is absent in fast freezing, but cooling and crystallization below −40 °C is lethal; it can cause cell damage. So, the optimum freezing rate is somewhere between these two. By the high rate of cooling, solidification of the system without crystal formation takes place. This amorphous ice state is called vitrification state. So, the optimum method is a multistage procedure for freezing involving slow cooling at the beginning until extracellular crystallization occurs. Then the optimum cell dehydration is realized by keeping the temperature constant. And fast cooling follows at the end until complete solidification takes place. Programmed

freezing prevents the critical volume contraction and intracellular ice crystallization. The storage of culture in the cryotank is the bigger aspect (Challen and Elliot 1986).

22.10.3.3 Freeze-Drying (Lyophilization)

Water inside the cell of microorganisms plays a very important role in various vital reactions, and removal of water halts both enzymatic and non-enzymatic reactions. During freeze-drying, water is removed by sublimation from the cell. In the process of freeze-drying, solvent (example—water) is reduced first by sublimation (primary drying) and then by desorption (secondary drying) to values that will no longer support biological growth or chemical reactions. Generally, it is assumed that cells in stationary growth phase are more tolerant to desiccation as compared to growing log phase cells. During starvation, cells can induce genes to encode transcription factors regulating the production of stress proteins, whereas reducing the pH of the growth medium can induce changes in the bacterial cell membrane fatty acid composition and initiate the production of acid shock proteins. Cells that are incapable of producing proteins due to desiccation damage may recover if additional stress response proteins are produced prior to drying.

Protective agents used in this technique before drying are known as lyoprotectants. Thus, various microorganisms can be preserved by this method with the help of lyoprotectants by pulling the water out using a vacuum (sublimation). Some important lyoprotectant are non-fat milk solids, serum, trehalose, glycerol, sucrose, betaine, adonitol, glucose, lactose, and polymers such as dextran and polyethylene glycol. These can be classified into two categories: (1) amorphous glass forming (example: carbohydrates, proteins, and polymers) and (2) eutectic crystallizing salts. The glass-forming additives have been shown to exert the highest protection during freeze-drying. The inert amorphous glass is also able to retain waste products released by the cells within the glass structure before freezing; therefore, waste products do not concentrate and initiate irreversible electrochemical changes on the plasma membrane during storage, whereas eutectic crystallizing salts form crystal structures as the crystallization point is reached. Each solute has different crystallization temperature. The formation of harmful salts or ice crystals can damage the cell membrane, thereby compromising the integrity of cell and allowing valuable cell contents to leak out of the cell after thawing. At the freezing point of water, ice crystals form, and remaining salts left in solution are concentrated around the ice crystals. Highly concentrated salt solutions in the interstitial region mixed with any substances released by the cells during freezing are considered detrimental to a cell as they cause irreversible damage to the cell membrane. The advantages of freeze-dried microorganisms are stabilized cell viability and also the ease of handling for shipment and storage costs. By this method, cultures can be stored for up to15–20 years (Fig. 22.4). Log phase cultures are lyophilized by suspending in lyophilization medium and later freeze-drying. Freeze-drying has some undesirable side effects, such as denaturation of sensitive proteins leading to decreased viability or activity. Not all bacteria can be freeze-dried (Miyamoto-Shinohara et al. 2000, 2006, 2008); generally, gram-positive bacteria show a far higher survival rate during freeze-

Fig. 22.4 Lyophilized ampoules and their storage chamber

drying than gram-negative bacteria. Recent advances in the field of freeze-drying have seen the development of freeze-dried products that contain exact numbers of freeze-dried cells. These products are used as reference materials for quality control purposes to enable accurate quantification of detection method efficiencies and quality control of growth media. Certain adverse conditions during microbial growth can induce tolerance responses. For example, lowering the pH during culturing can induce a greater protection to cells during freeze-drying.

22.11 Latest Techniques for Preservation

22.11.1 Sordelli's Method of Preservation

This is a method of preservation which is simpler than freeze-drying but as reliable as cryopreservation. This method can safely be used when the samples to be preserved are small in quantity. The culture should be preserved or incubated on solid medium for the required period. The inoculum is emulsified in a loopful of horse serum and is deposited on the inner wall of the small tube, which is inserted into another large tube. A small quantity of phosphorus pentoxide is placed at the bottom or the outer tube with the help of a glass rod. The inner tube must be placed in such a way that it is held over the bottom of the outer tube but not directly touching the chemical placed at the bottom. The outer tube is then connected to a vacuum pump, and after the air is removed, the outer tube is sealed. This tube containing the culture can be stored at room temperature away from light (Soriano 1970).

22.11.2 Vitrification

Rall and Fahy (1985) for the first time used vitrification method for the purpose of reproductive cryopreservation. This process protects cell from the damage caused due to the formation of ice crystals. In this process, cells are typically surrounded by

a vitrification solution which acts like a cryoprotective solution which turns to amorphous glass on cooling. This anti-freeze is a viscous solution which decreases the freezing temperature and prevents the onset of cryo-injury. Also samples are rapidly cooled which also eliminate the use of control cooling. The revival of culture is much simpler as it is directly warmed and then liquefied. Care must be taken that the samples do not crack, which could cause physical damage to the microbial culture. Also the sample must be washed to remove the toxic chemical of vitrifying mixture (Bhat et al. 2005).

For an effective cryopreservation, the solute of cryoprotectant must penetrate the cell in order to increase viscosity, thereby decreasing the freezing temperature. Cryoprotectant such as dimethyl sulfoxide is toxic at high concentrations. Vitrifying solution is comparatively better with the damage caused by the cryoprotective solutions as it has limited penetration inside the cell in addition to the toxicity effect of cryoprotectant. A study conducted by Leslie et al. (1995) on *Escherichia coli* DH5-α and *Bacillus thuringiensis* HD-1 showed an increased tolerance to freeze-drying when dried in the presence of the disaccharides trehalose and sucrose which has lowered the membrane phase transition temperature and protected protein structure in the dry state. Mixtures of cryoprotectants and ice blockers have enabled vitrification of a rabbit kidney to −135 °C. Upon rewarming, the kidney was successfully transplanted to rabbit, with total functionality and viability (Fahy et al. 2009).

22.11.3 Encapsulation

It is a technique where cells are embedded in a calcium alginate beads prior to cryopreservation. Use of this technique is not well documented for microorganisms. Its application has two main benefits: firstly, the water content of the cell may reduce by osmotic treatment or drying which decreases the prospect of ice damage or concentration effects during the cooling stage of cryopreservation procedure, and secondly, it allows cells to be easily handled and manipulated by providing a suspending matrix. Specimens are then rapidly "plunge" cooled with no need for control rate cooling (Sugiura et al. 2005).

22.11.4 Preservation Through Growth Substrate

For obligate pathogen or mutualistic microorganisms, preservation with their growth substrate or host has been applied for many years. For example, hemp seeds have been used to support members of the Chromista when cryopreserved. This approach has been used for microcyclic rust fungi *Puccinia spegazzinii* when the teliosphores were present on the petiole tissue. The alternative approach of cryopreservation has enormous potential for the large number of unculturable microorganisms, which can otherwise not be stored by genetic resource centers (Stomeo et al. 2009).

22.12 Registration of Elite Microbial Germplasm at ICAR-NBAIM

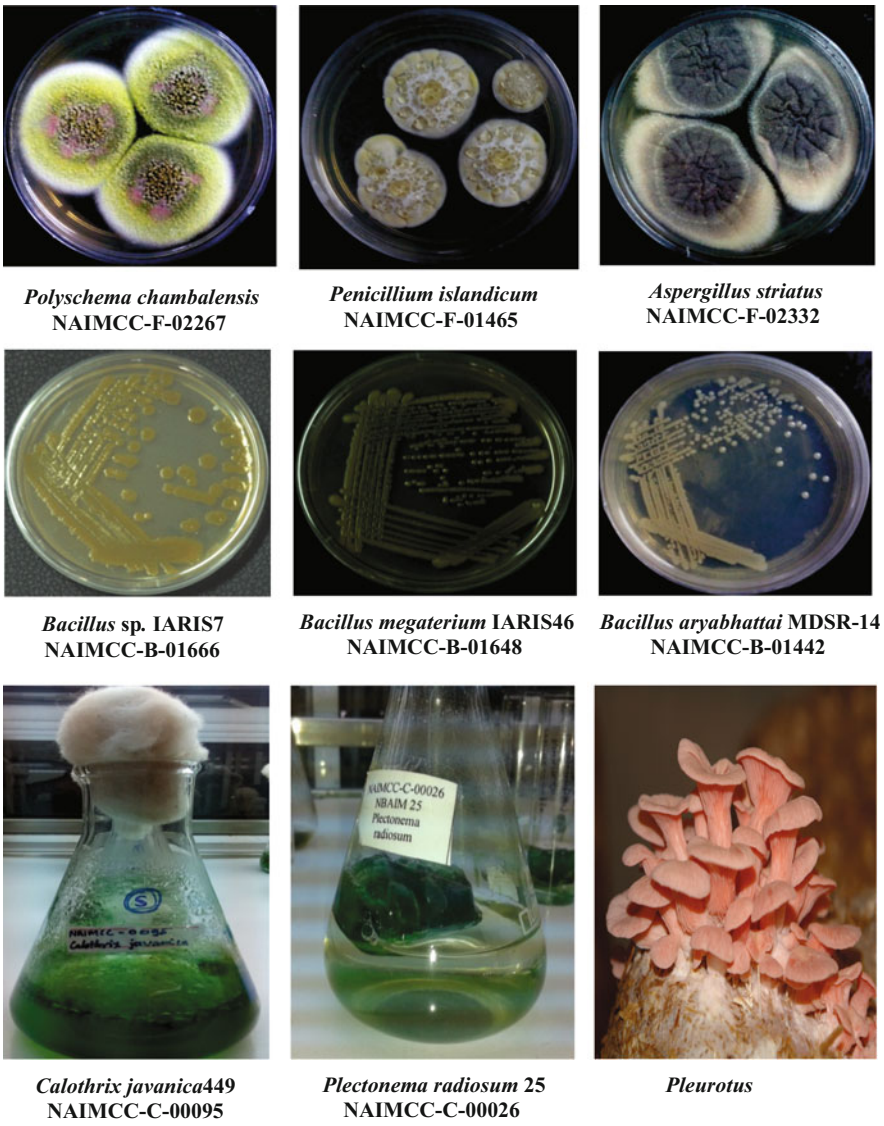
Registration of elite microbial germplasm is a new initiative taken up by the National Agriculturally Important Microbial Culture Collection (NAIMCC), ICAR-NBAIM, Mau. Germplasm that can be registered includes bacteria, fungi, cyanobacteria, and actinomycetes, having unique, distinguishable, and stable character and has potential proven attributes of academic, scientific, agricultural, industrial, and commercial importance (Fig. 22.5). Registration is done for a specific period of time 5 years. The passport data application submitted for registration of germplasm should include all the authentic details regarding taxonomy, geographical location, method of preservation, risk group, and its uniqueness. The details of the same can also be found on http://www.mgrportal.org.in/Registration.html. Deposition of culture should be supported by sufficient evidence of uniqueness like publication in standard peer-reviewed journal and certified evaluation data for at least 3 years under AICRP trial/Nursery test for three locations under any other relevant system. Plasmid which is a part of the microorganism will not be covered under "kind of microorganisms". Contaminated microorganisms or those which do not have unique properties are not accepted. Microbial Germplasm Registration Committee is set up to check the authenticity and uniqueness of the deposit material.

22.12.1 Nodal Agency

ICAR-NBAIM, Maunath Bhanjan, Uttar Pradesh, is the nodal agency for registration of germplasm. The application concerning the registration should be addressed to the Director, NBAIM, along with the microbial cultures and other relevant documents. The member secretary of the committee forwards the proposal to reviewers for thorough examination and critical comments.

22.12.2 Microbial Germplasm Registration Committee (MGRC)

On receipt of report of reviewers, the proposal is critically reviewed by MGRC. It is a committee constituted under the chairmanship of Deputy Director General (Crop Sciences), Indian Council of Agricultural Research. Director, ICAR-NBAIM, will act as a permanent member with a senior level scientist as member secretary. Specialist experts can be called on requirement for a group of microorganisms with reference to the material under consideration. After reviewing by the nodal centre, the proposal completed in all respects with the comment(s) of the concerned authority is put before the registration committee for consideration. The whole process of reviewing and decision of MGRC is to be completed in 1 year from the receipt of complete application.



Polyschema chambalensis **NAIMCC-F-02267**

Penicillium islandicum **NAIMCC-F-01465**

Aspergillus striatus **NAIMCC-F-02332**

Bacillus **sp. IARIS7 NAIMCC-B-01666**

Bacillus megaterium **IARIS46 NAIMCC-B-01648**

Bacillus aryabhattai **MDSR-14 NAIMCC-B-01442**

*Calothrix javanica***449 NAIMCC-C-00095**

Plectonema radiosum **25 NAIMCC-C-00026**

Pleurotus

Fig. 22.5 Different microbial cultures in NAIMCC

22.12.3 Validity of Registration

The period for validity of registration shall be for 5 years from the date of registration; after that the registered germplasm would be a national sovereign property.

22.12.4 Notification of Registered Germplasm

All germplasm material approved for registration would be officially communicated to the applicants along with Registration Number. A certificate to this effect will also be issued to the applicant. A brief description should be published in appropriate periodicals, such as:

- Any NAAS-rated journal such as Indian Journal of Microbiology—published by Association of Microbiologist of India, etc.
- ICAR-NBAIM Annual Report, ICAR-NBAIM, Maunath Bhanjan, UP. 275103
- ICAR News—published by the Publication and Information Division, Krishi Anusandhan Bhavan, ICAR, New Delhi—110 012
- ICAR-NBAIM Website http://www.nbaim.org /www.mgrportal.org.in

22.12.5 Conservation, Maintenance, and Sustainable Utilization of Registered Germplasm

- Registered germplasm will be conserved in the NAIMCC in glycerol stock and/or mineral oil and/or liquid nitrogen and/or lyophilized forms as far as possible.
- The novel gene will be conserved in the gene bank of ICAR-NBAIM.
- The institution associated with the development of the germplasm is also to be mandated with the maintenance of working stock of germplasm for supply to users.

22.13 Conclusion

Microorganisms play an important role in biogeochemical transformation and are utilized in pharmaceutical, biotechnology, and food industries. The preservation of microorganisms is of paramount importance keeping in view the role played by them in various sectors of the economy. Culture collections are a storehouse of biological resources which have to be preserved for posterity so that in future they can be further screened, genetically improved, characterized, and used for production of valuable products. They provide high-quality biological resources for research, teaching, and quality control in industry.

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References

Allgaier M, Brückner S, Jaspers E, Grossart H-P (2007) Intra- and inter-lake variability of free-living and particle-associated Actinobacteria communities. Environ Microbiol 9:2728–2741. doi:10.1111/j.1462-2920.2007.01385.x

Bhat SN, Sharma A, Bhat SV (2005) Vitrification and glass transition of water: insights from spin probe ESR. Phys Rev Lett 95:235702. doi:10.1103/PhysRevLett.95.235702

Burdsall HH Jr, Dorworth EB (1994) Preserving cultures of wood-decaying Basidiomycotina using sterile distilled water in cryovials. Mycologia 86:275–280

Castellani A (1963) Further researches on the long viability and growth of many pathogenic fungi and some bacteria in sterile distilled water. Mycopathol Mycol Appl 20:1–6. doi:10.1007/BF02054872

Challen MP, Elliot TJ (1986) Polypropylene straw ampoules for the storage of microorganisms in liquid nitrogen. J Microbiol Methods 5:11–22. doi:10.1016/0167-7012(86)90019-9

Cockell CS, Jones HL (2009) Advancing the case for microbial conservation. Oryx 43:520–526

Diogo HC, Sarpieri A, Pires MC (2005) Fungi preservation in distilled water. An Bras Dermatol 80:591–594

Ellis JJ (1979) Preserving fungus strains in sterile water. Mycologia 71:1072–1075. doi:10.2307/3759297

Fahy GM, Wowk B, Pagotan R, Chang A, Phan J, Thomson B, Phan L (2009) Physical and biological aspects of renal vitrification. Organogenesis 5:167–175

Gerrath JF, Gerrath JA, Matthes U, Larson DW (2000) Endolithic algae and cyanobacteria from cliffs of the Niagara Escarpment, Ontario, Canada. Can J Bot 78:807–815. doi:10.1139/b00-042

Gherna RL (2009) Culture preservation, Encyclopedia of industrial biotechnology. Wiley, pp. 153–161

Homolka L (2014) Preservation of live cultures of basidiomycetes—recent methods. Fungal Biol 118:107–125. doi:10.1016/j.funbio.2013.12.002

Kulkarni G, Chitte R (2015) Preservation of thermophilic bacterial spores using filter paper disc techniques. J Bioprocess Biotechniques 5:223. doi:10.4172/2155-9821.1000223

Leslie SB, Israeli E, Lighthart B, Crowe JH, Crowe LM (1995) Trehalose and sucrose protect both membranes and proteins in intact bacteria during drying. Appl Environ Microbiol 61:3592–3597

Liao CH, Shollenberger LM (2003) Survivability and long-term preservation of bacteria in water and in phosphate-buffered saline. Lett Appl Microbiol 37:45–50

Malik KA (1990) A simplified liquid-drying method for the preservation of microorganisms sensitive to freezing and freeze-drying. J Microbiol Methods 12:125–132. doi:10.1016/0167-7012(90)90022-X

Marx DH, Daniel WJ (1976) Maintaining cultures of ectomycorrhizal and plant pathogenic fungi in sterile water cold storage. Can J Microbiol 22:338–341. doi:10.1139/m76-051

Miyamoto-Shinohara Y, Imaizumi T, Sukenobe J, Murakami Y, Kawamura S, Komatsu Y (2000) Survival rate of microbes after freeze-drying and long-term storage. Cryobiology 41:251–255

Miyamoto-Shinohara Y, Sukenobe J, Imaizumi T, Nakahara T (2006) Survival curves for microbial species stored by freeze-drying. Cryobiology 52:27–32

Miyamoto-Shinohara Y, Sukenobe J, Imaizumi T, Nakahara T (2008) Survival of freeze-dried bacteria. J Gen Appl Microbiol 54:9–24

Olembo R, Hawksworth D (1991) Importance of microorganisms and invertebrates as components of biodiversity. Biodiversity of microorganisms and invertebrates: its role in sustainable agriculture. In: Proceedings of the First Workshop on the Ecological Foundations of Sustainable Agriculture (WEFSA 1). CAB International, London, pp 7–15

Overmann J (2015) Significance and future role of microbial resource centers. Syst Appl Microbiol 38:258–265

Perkins DD (1962) Preservation of Neurospora stock cultures with anhydrous silica gel. Can J Microbiol 8:591–594

Pichugin YI (1993) Results and perspectives in searching of new endocellular cryoprotectants. Probl Cryobiol 2:3–8
Prakash O, Nimonkar Y, Shouche YS (2013) Practice and prospects of microbial preservation. FEMS Microbiol Lett 339:1–9
Rall WF, Fahy GM (1985) Ice-free cryopreservation of mouse embryos at −196 degrees C by vitrification. Nature 313:573–575
Richter DL, Bruhn JN (1989) Revival of saprotrophic and mycorrhizal basidiomycete cultures from cold storage in sterile water. Can J Microbiol 35:1055–1060. doi:10.1139/m89-176
Richter DL, Kangas LC, Smith JK, Laks PE (2010) Comparison of effectiveness of wood decay fungi maintained by annual subculture on agar and stored in sterile water for 18 years. Can J Microbiol 56:268–271. doi:10.1139/W10-001
Sakane T, Fukuda I, Itoh T, Yokota A (1992) Long-term preservation of halophilic archaebacteria and thermoacidophilic archaebacteria by liquid drying. J Microbiol Methods 16:281–287. doi:10.1016/0167-7012(92)90080-N
Scharf CA (2009) Extrasolar planets and astrobiology. University Science Books, Sausalito, CA
Sharma A, Shouche Y (2014) Microbial culture collection (MCC) and International Depositary Authority (IDA) at National Centre for Cell Science, Pune. Ind J Microbiol 54:129–133. doi:10.1007/s12088-014-0447-y
Shridhar BS (2012) Review: nitrogen fixing microorganisms. Int J Microbiol Res 3:46–52. doi:10.5829/idosi.ijmr.2012.3.1.61103
Smith D, Kirsop BE, Snell JJS (1984) Maintenance of microorganisms: a manual of laboratory methods. Academic, London
Smith D (2003) Culture collections over the world. Int Microbiol 6:95–100. doi:10.1007/s10123-003-0114-3
Smith D, Ryan MJ, Stackebrandt E (2008) The ex situ conservation of microorganisms: aiming at a certified quality management, Biotechnology. EOLSS Publisher, Oxford
Smith D, Ryan M (2012) Implementing best practices and validation of cryopreservation techniques for microorganisms. Scientific World Journal 2012:805659. doi:10.1100/2012/805659
Soriano S (1970) Sordelli's method for preservation of microbial cultures by desiccation in vacuum. In Int Conf Cult Collect Tokyo 1968 Cult Collect Microorg, pp 298–305
Stomeo F, Portillo M, Gonzalez J (2009) Assessment of bacterial and fungal growth on natural substrates: consequences for preserving caves with prehistoric paintings. Curr Microbiol 59:321–325. doi:10.1007/s00284-009-9437-4
Sugiura S, Oda T, Izumida Y, Aoyagi Y, Satake M, Ochiai A, Ohkohchi N, Nakajima M (2005) Size control of calcium alginate beads containing living cells using micro-nozzle array. Biomaterials 26:3327–3331. doi:10.1016/j.biomaterials.2004.08.029
Tommerup IC (1988) Long-term preservation by L-drying and storage of vesicular arbuscular mycorrhizal fungi. Trans Br Mycol Soc 90:585–591. doi:10.1016/S0007-1536(88)80063-9
Uruburu F (2003) History and services of culture collections. Int Microbiol 6:101–103. doi:10.1007/s10123-003-0115-2
Vincent WF (2000) Evolutionary origins of Antarctic microbiota: invasion, selection and endemism. Antarct Sci 12:374–385. doi:10.1017/S0954102000000420
Weinbauer MG, Rassoulzadegan F (2007) Extinction of microbes: evidence and potential consequences. Endangered Species Res 3:205–215

Chapter 23
Biosurfactants in Microbial Enhanced Oil Recovery

Sunita J. Varjani

Abstract Industrial sustainability has pushed biosurfactants as a tool for many companies. Biosurfactants offer possibility of replacing chemical surfactants, produced from nonrenewable resources and/or cheap renewable feedstocks. Biosurfactants are also attractive as they are ecofriendly. The most promising biosurfactants nowadays are glycolipids, lipopeptides, phospholipids, fatty acids/natural lipids, polymeric surfactants, and particulate biosurfactants. Biosurfactants find utility in various industrial sectors such as petroleum, food, beverage, cosmetics, detergents, pharmaceuticals, and environmental protection and management. This chapter highlights prospects for commercial exploration of a new generation microbial biosurfactants as a tool in bioremediation and microbial enhanced oil recovery (MEOR).

23.1 Introduction

Surface tension (SFT) of a liquid can be defined as force per unit length on surface which opposes expansion of surface area. Surfactants in solution have ability to gather at interfaces between immiscible phases which reduce SFT and interfacial tension (IFT). Surfactants are amphiphilic molecules with a hydrophobic and hydrophilic part (Hoskova et al. 2013; Varjani et al. 2014). Chemical surfactants have a major impact on our lives. Companies using chemical surfactants are now looking for alternatives, i.e., surfactant molecules produced principally by microorganisms (Varjani 2014). These molecules have advantage that they are stable at relatively harsh environmental conditions, *viz*., pH, temperature, and salinity; low or no toxicity; and are readily biodegradable in environment if, or when, discharged (Banat 1995; Henkel et al. 2012).

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Hydrocarbons, as hydrophobic organic chemicals, exhibit limited solubility in water and tend to partition to soil matrix (Varjani et al. 2013). This partitioning can account for as much as 90–95 % or more of the total contaminant mass. As a consequence, hydrocarbon contaminants exhibit moderate to poor recovery by physicochemical treatments, limited bioavailability to microorganisms, and limited availability to oxidative and reductive chemicals when applied to in situ and/or ex situ applications (Satpute et al. 2010; Vasconcellos et al. 2011; Varjani and Upasani 2012; Pacwa-Płociniczak et al. 2014). Naturally produced surface-active compounds of biological origin are called biosurfactants. Biosurfactants act through changing surface-active phenomena, such as reducing surface and interfacial tensions, thereby increasing surface area of hydrophobic substrates; wetting and penetrating actions; spreading, hydrophilicity, and hydrophobicity actions; enhancement of bioremediation rate, oil recovery, and microbial growth by increasing availability of hydrophobic substrates; metal sequestration; and antimicrobial action (Banat 1995; Jain et al. 2011; Varjani et al. 2014). Several bacteria use hydrocarbons as a sole food source and produce various products like gases, biosurfactants (BS), biopolymers, solvents, and acids (Pacwa-Plociniczak et al. 2011). These products help in enhancing bioremediation and oil recovery (Chaillan et al. 2004; Henkel et al. 2012; Varjani et al. 2015). This chapter throws light on application of biosurfactants in solving petroleum industry problems such as bioremediation and microbial enhanced oil recovery (MEOR). This chapter also represents biosurfactants as a tool to study potentials of microbes in petrochemical industry.

23.2 History and Key Mechanisms for MEOR

Successful Microbial Enhanced Oil Recovery (MEOR) field trials have been conducted in the USA, Russia, Romania, China, Australia, Germany, Hungary, India, Malaysia, Peru, and Poland (Varjani 2014). For MEOR, Institute of Reservoir Studies (IRS), Oil and Natural Gas Corporation (ONGC), and The Energy and Resources Institute (TERI) have developed a bacterial consortium S2, surviving at 90 °C, up to 140 kg/cm^2 pressure and 4–8 % (w/v) NaCl concentration. Three fold increase in oil recovery has been reported by injecting selected microbes into selected oil wells of ONGC in Ahmedabad and Mehsana assets in Gujarat state (http://www.otbl.co.in/meor.html).

Microorganisms have developed different strategies to overcome problem of low bioavailability of hydrophobic substrates using three mechanisms (a) high-affinity uptake systems (Miyata et al. 2004), (b) adhesion to solid substrate, e.g., formation of a biofilm (Eriksson et al. 2002), and (c) production of biosurfactant (Lang 2002; Henkel et al. 2012; Varjani et al. 2014). Biosurfactants are surface-active agents of microbial origin which enhance water solubility and subsequent bacterial degradation of organic contaminants as well as enhance oil recovery

(Kuiper et al. 2004). Biosurfactants are produced either on microbial cell surface or extracellular excreted (Pacwa-Plociniczak et al. 2011; Markande et al. 2013).

23.3 Biosurfactant Classification

Biosurfactants with different chemical structures and properties have been reported from various microorganisms isolated from petroleum contaminated sites (Desai and Banat 1997; Henkel et al. 2012; Vijaya et al. 2013; Varjani et al. 2014). Biosurfactants are broadly low molecular weight glycolipids/lipopeptides or high molecular weight polysaccharides/proteins molecules. Some examples for biosurfactant producing hydrocarbon degrading microorganisms includes *Pseudomonas aeruginosa* (rhamnolipids), *Candida apicola* (sophorolipids), *Rhodococcus erythropolis* (trehalose dimyeolipids), *Bacillus* sp. (lichenysins), and *Bacillus subtilis* (surfactin) (Desai and Banat 1997). The hydrophilic moiety usually consists of amino acids, ions (anions or cations), or polysaccharides.The hydrophobic moiety consists of saturated or unsaturated fatty acids. Based on structural features, biosurfactants are classified in following types: (a) glycolipids; (b) lipoproteins or lipopeptides; (c) phospholipids, fatty acids, or natural lipids; (d) polymeric biosurfactants; and (e) particulate biosurfactants (Desai and Banat 1997; Bodour et al. 2004; Thavasi et al. 2011).

23.3.1 Glycolipids

Glycolipids are sugar-containing lipids in which a carbohydrate moiety is linked to a fatty acid moiety (aliphatic acids or hydroxyl aliphatic acids) (Müller and Hausmann 2011). The best known glycolipids are rhamnolipids, sophorolipids, and trehalolipids (Desai and Banat 1997; Henkel et al. 2012).

23.3.1.1 Rhamnolipids

They are the most common glycolipids mainly produced by *P. aeruginosa*. Rhamnolipids (RLs) are formed by one or two rhamnose (hydrophilic) molecules linked to one or two fatty acids (hydrophobic), which are saturated or unsaturated alkyl chains (Haba et al. 2003; Lourith and Kanlayavattanakul 2009). RLs are secondary metabolites, and their production starts with onset of stationary phase. They play an important role in motility, biofilm formation, and solubilization of hydrocarbon pollutants. RLs were first isolated from *P. aeruginosa*. They are produced in relatively high amount in relatively short incubation periods exhibiting relatively high surface activities. *P. aeruginosa* is known to have approximately 6.29 Mbp genome; its genes are involved in catabolism, transport, and interchange

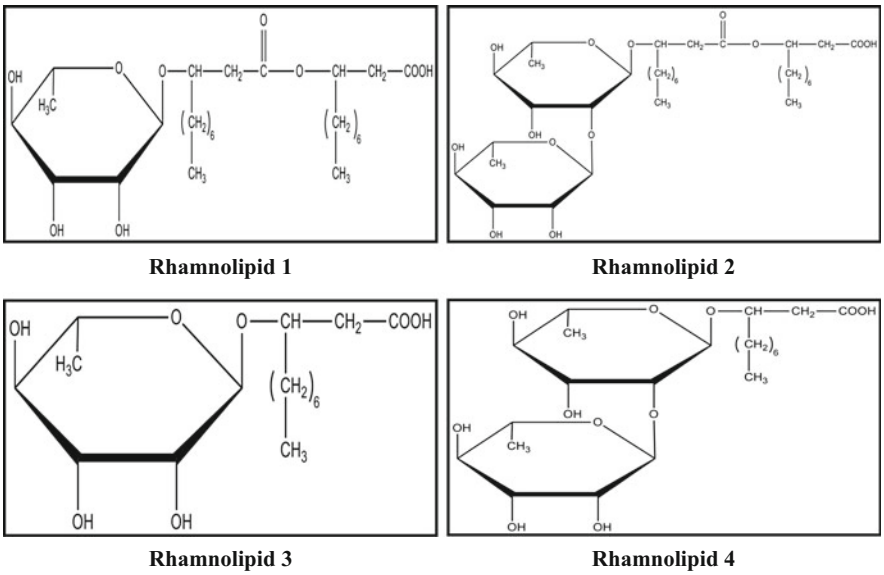

Fig. 23.1 Structures of Rhamnolipids from *P. aeruginosa* (Source: Lang and Wullbrandt 1999)

of organic compounds as well as four potential chemotaxis systems. Diverse metabolic capabilities of *P. aeruginosa* make it important to utilize a wide variety of substrates, some of which are not easily degraded by other organisms (Jarvis and Johnson 1949).

Twenty eight homologues of RLs have been identified, and mixtures of these homologues can be produced by a single strain of *P. aeruginosa* (Abalos et al. 2001). However, types of RLs produced depend on bacterial strain, carbon source used, and process strategy. Four homologues of rhamnolipids are most predominant. Principal RLs mono-rhamno-di-lipidic congener (R1) and di-rhamno-di-lipidic congener (R2) consist of one or two L-rhamnose units and two units of β-hydroxydecanoic acid, while mono-rhamno-mono-lipidic congener (R3) and di-rhamno-mono-lipidic congener (R4) consist of one or two L-rhamnose and one unit of β-hydroxydecanoic acid (Lang and Wullbrandt 1999). These four compounds are commonly referred to as R1–R4 rhamnolipids and are shown in Fig. 23.1.

23.3.1.2 Sophorolipids and Trehalolipids

Sophorolipids and trehalolipids are similar in structure (Cooper and Zajic 1980). Sophorolipids consist of a dimeric sophorose molecule which is linked to a long chain hydroxy fatty acid and are mainly produced by yeasts *Torulopsis bombicola* and *T. petrophilum* (Desai and Banat 1997). Trehalolipids are characterized by

disaccharide trehalose linked at C_6 to two β-hydroxy-branched fatty acids. They have been isolated from several strains of genera *Arthrobacter*, *Mycobacterium*, *Brevibacterium*, *Corynebacterium*, *Candida*, and *Nocardia*. Trehalolipids produced by these organisms differ in structure of fatty acids and degree of unsaturation (Cooper and Zajic 1980; Desai and Banat 1997; Lourith and Kanlayavattanakul 2009).

23.3.2 Lipoproteins or Lipopeptides

Lipopeptides are characterized by hydrophilic amino acid chain (peptide) which is linked to fatty acid. The peptide is either linear or cyclic. Surfactin is a popular lipopeptide mainly produced by *Bacillus* sp. which has a cyclic peptide and β-hydroxyl fatty acid (Davis et al. 1999). The cyclic peptide consists of seven amino acids, which may differ in sequence, but in all cases it is composed of five lipophilic amino acids and two negatively charged hydrophilic amino acids (Singh and Cameotra 2004; Buchoux et al. 2008). The length and structure of fatty acids may also differ. Generally, fatty acid consists of 13–16 carbon atoms and may be branched (Akpa et al. 2001).

23.3.3 Phospholipids, Fatty Acids, or Natural Lipids

Phospholipids, neutral lipids, and some fatty acids are components of cell structures and have surface activity usually associated with biosurfactants. Flavolipids are biosurfactant of acid tail groups. Phospholipids are probably best known as a major constituent of all cell membranes. They have been produced by certain strains of genera *Acenitobacter*, *Aspergillus*, and *Thiobacillus* and consist of a fatty acid linked to cationic phosphate group. Molecular weight of this type of biosurfactants is approximately 500–3000 Dalton (Varjani 2014).

23.3.4 Polymeric and Particulate Surfactants

Polymeric biosurfactants have molecular weight greater than 50,000 Dalton. Biopolymers consist of a polysaccharide backbone to which fatty acid side chains are covalently linked. The most intensively studied polymeric biosurfactants are emulsan and liposan and are produced by *Acinetobacter calcoaceticus* and *Candida lipolytica*, respectively. They are generally more effective in stabilizing emulsions of oil in water but do not have capacity to lower surface tension (Desai and Banat 1997). Microorganisms shown to produce biosurfactants are represented in Table 23.1.

Table 23.1 Major biosurfactant classes and microorganisms (Karanth et al. 2007)

Biourfactant class	Microorganisms
Glycolipids	
Rhamnolipids	*Pseudomonas aeruginosa*
Trehalose lipids	*Rhodococcus erythropolis*, *Arthrobacter* sp.
Sophorolipids	*Candida bombicola*, *Candida apicola*
Lipoproteins or Lipopeptides	
Surfactin/iturin/fengycin	*Bacillus subtilis*
Viscosin	*Pseudomonas fluorescens*
Lichenysin	*Bacillus licheniformis*
Phospholipids, Fatty acids or Natural lipids	
Surface-active antibiotics	*Acinetobacter* sp., *Corynebacterium lepus*
Gramicidin	*Brevibacterium brevis*
Polymixin	*Bacillus polymyxa*
Fatty acids or natural lipids	*Corynebacterium insidibasseosum*
Polymeric biosurfactants	
Emulsan	*Acinetobacter calcoaceticus*
Alasan	*Acinetobacter radioresistens*
Liposan	*Candida lipolytica*
Particulate biosurfactants	*Acinetobacter calcoaceticus, Cyanobacteria*

23.4 Biosurfactant Applications

The concept of environmental protection leads towards the consideration of biosurfactants as alternative to synthetic surfactants (Sarachat et al. 2010). BS finds utility in various industries, *viz*., petrochemical, food, beverage, cosmetics, detergents, pharmaceuticals, paint, agriculture, mining, environmental protection and management, etc. They can be used as emulsifiers, de-emulsifiers, wetting agent, dispersing agent, foaming agents, detergents, and active food ingredients (Banat 1995; Deleu and Paquot 2004; Thavasi et al. 2011; Rikalovic *et al*. 2012). Presently, biosurfactants are mainly used in petroleum industry for enhanced oil recovery and hydrocarbon remediation (Cameotra and Makkar 1998). Here, applications of biosurfactants in petroleum industry are discussed. Biosurfactants produced from industrial wastes and by-products in particular are used (a) for bulk production of petroleum products, (b) bioremediation of environmental pollutants including oil spills, biodegradation, and detoxification of industrial effluents, and (c) Microbial Enhanced Oil Recovery (MEOR). Biosurfactants increase growth of microbes through enhanced bioavailability of hydrocarbons thereby enhancing degradation of contaminants by indigenous microbes (Pacwa-Plociniczak et al. 2011).

23.4.1 Biosurfactants: A Tool for Bioremediation

Bioremediation technologies have replaced physical and chemical remediation processes for cleanup of contaminated soils (Owsianiak et al. 2009; Juwarkar et al. 2010; Cerqueira et al. 2011; Varjani and Upasani 2012). Bioremediation of petroleum hydrocarbons in contaminated soil was initially developed for oil industry more than three decades ago to search for cleanup method of oil sludge from their operations, immobilizing pollutants or transforming them into non-hazardous products for environment (Hazen et al. 2003; Varjani et al. 2013). Since then application of this technology has increased and has been applied through different approaches in heterogeneous environments (Aislabie et al. 2006). The use of surfactants in situations of oil contamination may have a stimulatory, inhibitory, or neutral effect on bacterial degradation of oil components (Jain et al. 2011). Introduction of external surfactants as main components of oil spill dispersants influence alkane degradation rate (Rahman et al. 2003). Certain microorganisms such as *B. subtilis, P. aeruginosa*, and *T. bombicola* have been reported to utilize crude oil and hydrocarbons as sole carbon sources for biosurfactant production and can be a useful mechanism for oil spill cleanup as well as oil sludge removal (Mulligan et al. 2001; Das and Mukherjee 2007). Exxon Valdez oil-tanker spill was successfully bioremediated by using biosurfactants (Mulligan et al. 2001). Varjani et al. (2015) performed experiment (3 % v/v crude oil as sole carbon source, pH 7.2, 37 °C, 75 days at 180 rpm with 2 % inoculum) on biodegradation of crude oil (ONGC, India), using consortium of six indigenous bacterial isolates. They reported 81.66 % degradation of n-paraffins (C8–C36+) and 83.49 % degradation of C8–C35.

23.4.2 Biosurfactants: A Tool for MEOR

Microbial-enhanced oil recovery (MEOR) processes were earlier known as "microbial increased oil recovery" (MIOR). MEOR technique involves processes where both microorganism(s) and/or their products are used to recover oil from individual wells or entire reservoirs. MEOR activities were commenced far back since 1926 in most developed countries and is ongoing till date. The alarming rate of decrease in supply of petroleum and its products makes MEOR useful which will stabilize future worldwide oil production and ensure adequate energy supply (Osunde and Balogun 2013).

Biosurtactants play a crucial role to enhance bioavailability of hydrocarbon pollutants, and it is very well studied. These surface-active agents may stimulate dissolution or desorption rates, solubilization, or even emulsification of hydrocarbons (Banat 1995; Henkel et al. 2012; Varjani et al. 2014). Injection of microorganisms, nutrients, or microbial byproducts (gases, biosurfactants, biopolymers, solvents, and acids) can take place (a) in individual wells separately through the

cyclic injection method (bio-huff and puff method), (b) in conjunction with a water flood (the field flood method), and (c) through either cyclic injection or field flooding (well-prep treatment). Mechanisms responsible for oil release from porous media involve processes such as (a) dissolution of inorganic carbonates by bacterial metabolites, (b) production of bacterial gases which decrease viscosity of oil thereby promoting its flow, (c) production of surface-active substances or wetting agents by some bacteria, and (d) high affinity of bacteria for solids, later attached to crowd off oil films (Lazar et al. 2007).

Various researchers have performed work on MEOR, *viz*., Abu-ruwaida et al. 1991; Suthar et al. 2008; Adelzadeh et al. 2010; Rabiei et al. 2013. Varjani (2014) has reported 8.82 % enhancement of oil recovery as % of residual oil saturation through ex situ bioaugmentation with rhamnolipid biosurfactant produced by *P. aeruginosa* NCIM 5514.

23.5 Conclusion

An increase in concern about environmental protection and management has caused the consideration of biosurfactants as alternative source for synthetic surfactants. The use of biosurfactants in petroleum industries show significant advantage over chemically synthesized surfactants such as lower toxicity, enhanced biodegradability, enhanced foaming capacity, high specificity, better environmental compatibility, and ability to be synthesized from renewable feedstocks. Biosurfactants serve as a potential tool to solve two burning issues of petroleum industry, *viz*., petroleum hydrocarbon pollution and increase of fuel prize (petrol, diesel, etc). Biosurfactants can be used as a tool to understand the ability of microbes in bioremediation and MEOR.

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References

Abalos A, Pinazo A, Infante MR, Casals M, García F, Manresa A (2001) Physicochemical and antimicrobial properties of new rhamnolipids produced by *Pseudomonas aeruginosa* AT10 from soybean oil refinery wastes. Langmuir 17:1367–1371

Abu-ruwaida AS, Banat IM, Haditirto S, Salem A, Kadri M (1991) Isolation of biosurfactant-producing bacteria product characterization and evaluation. Acta Biotechnologica 11:315–324

Adelzadeh MR, Roostaazad R, Kamali MR, Lotfabad TB (2010) A technical feasibility analysis to apply *Pseudomonas aeroginosa* MR01 biosurfactant in microbial enhanced oil recovery of low-permeability carbonate reservoirs of Iran. Transactions C: Chem Chem Eng 17:46–54

Aislabie J, Saul D, Foght J (2006) Bioremediation of hydrocarbon-contaminated polar soils. Extremophiles 10:171–179

Akpa E, Jacques P, Wathelet B, Paquot M, Fuchs R, Budzikiewicz H, Thonart P (2001) Influence of culture conditions on lipopeptide production by *Bacillus subtilis*. App Biochem Biotechnol 93:551–556

Banat IM (1995) Biosurfactants production and possible uses in microbial enhanced oil recovery and oil-pollution remediation: a review. Bioresour Technol 51:1–12

Bodour AA, Guerrero-Barajas C, Jiorle BV, MalcoMson ME, Paull AK, Somogyi A, Trinh LN, Bates RB, Maier RM (2004) Structure and characterization of flavolipids, a novel class of biosurfactants produced by *Flavobacterium sp*. strain MTN11. Appl Environ Microbiol 70:114–120

Buchoux S, Lai-Kee-Him J, Garnier M, Tsan P, Besson F, Brisson A, Dufourc EJ (2008) Surfactin-triggered small vesicle formation of negatively charged membranes: a novel membrane-lysis mechanism. Biophysical J 95:3840–3849

Cameotra SS, Makkar RS (1998) Synthesis of biosurfactants in extreme conditions. Appl Microbiol Biotechnol 50:520–529

Cerqueira VS, Hollenbach EB, Maboni F, Vainstein M, Camargo F, Do-Carmo RPM, Bento FM (2011) Biodegradation potential of oily sludge by pure and mixed bacterial cultures. Bioresour Technol 102:11003–11010

Chaillan F, LeFlèche A, Bury E, Phantavong YH, Grimont P, Saliot A, Oudot J (2004) Identification and biodegradation potential of tropical aerobic hydrocarbon degrading organisms. Res Microbiol 55:587–595

Cooper DG, Zajic JE (1980) Surface active compounds from microorganisms. Adv Appl Microbiol 26:229–256

Das K, Mukherjee AK (2007) Crude petroleum-oil biodegradation efficiency of *Bacillus subtilis* and *Pseudomonas aeruginosa* strains isolated from a petroleum-oil contaminated soil from North-East India. Bioresour Technol 98:1339–1345

Davis DA, Lynch HC, Varley J (1999) The production of surfactin in batch culture by *Bacillus subtilis* ATCC 21332 is strongly influenced by the conditions of nitrogen metabolism. Enz Microbial Technol 25:322–329

Deleu M, Paquot M (2004) From renewable vegetables resources to microorganisms: new trends in surfactants. Comptes Rendus Chimie 7:641–646

Desai JD, Banat IM (1997) Microbial production of surfactants and their commercial potential. Microbiol Mol Bio Rev 61:47–64

Eriksson MM, Dalhammar G, Mohn WW (2002) Bacterial growth and biofilm production on pyrene. FGMS Microbiol Eco 40:21–27

Haba E, Abalos A, Jáuregui O, Espuny MJ, Manres A (2003) Use of Liquid Chromatography-Mass Spectroscopy for studying the composition and properties of rhamnolipids produced by different Strains of *Pseudomonas aeruginosa*. J. Surfactants Deterg 6:155–161

Hazen TC, Tien AJ, Worsztynowicz A, Altman DJ, Ulfig K, Manko T (2003) Biopiles for remediation of petroleum-contaminated soils: a polish case study. The utilization of bioremediation to reduce soil contamination: problems and solutions (NATO science series) 19:229–246

Henkel M, Müller MM, Kügler JH, Lovaglio RB, Contiero J et al (2012) Rhamnolipids as biosurfactants from renewable resources: concepts for nextgeneration rhamnolipid production. Process Biochem 47:1207–1219

Hoskova M, Schreiberova O, Jezdik R, Chudoba J, Masak J, Sigler K, Rezanka T (2013) Characterization of rhamnolipids produced by non-pathogenic *Acinetobacter* and *Enterobacter* bacteria. Bioresour Technol 130:510–516

http://www.otbl.co.in/meor.html. Collected 25 Dec 2015

Jain PK, Gupta VK, Gaur RK, Lowry M, Jaroli DP, Chauhan UK (2011) Bioremediation of petroleum oil contaminated soil and water. Res J Environ Toxicol 5:1–26

Jarvis FG, Johnson MJ (1949) A glyco-lipid produced by *Pseudomonas aeruginosa*. J Am Chem Soc 71:4124–4126

Juwarkar AA, Singh SK, Mudhoo A (2010) A comprehensive overview of elements in bioremediation. Env Sci Biotechnol 9:215–288
Karanth NGK, Deo PG, Vernanadig NK (2007) Microbial production of biosurfactants and their importance. http://www.iisc.ernet.in/currsci/jul10/articles19.htm (Collected: 27.12.2015)
Kuiper I, Lagendijk EL, Bloemberge GV, Lugtenberg BJ (2004) Rhizoremediation: a beneficial plant–microbe interaction. MPMI 17:6–15
Lang S (2002) Biological amphiles (microbial biosurfactants). COCIS 7:12–20
Lang S, Wullbrandt D (1999) Rhamnose-lipids—biosynthesis, microbial production and application potential. Appl Microbiol Biotechnol 51:22–32
Lazar I, Petrisor IG, Yen TE (2007) Microbial enhanced oil recovery (MEOR). Petroleum Sci Technol 25:1353–1366
Lourith N, Kanlayavattanakul M (2009) Natural surfactants used in cosmetics: glycolipids. Int J Cosmetic Sci 29:225–261
Markande AR, Acharya SR, Nerurkar AS (2013) Physicochemical characterization of thermostable glycoprotein bioemulsifier from *Solibacillus silvestris* AM1. Process Biochem 48:1800–1808
Miyata N, Iwhori K, Foght JM, Gray MR (2004) Saturable, energy dependent uptake of phenanthrene in aqueous phase by Mycobacterium sp. strain RJG II-135. Appl Environ Microbiol 70:363–369
Müller MM, Hausmann R (2011) Regulatory and metabolic network of rhamnolipid biosynthesis: traditional and advanced engineering towards biotechnological production. Appl Microbiol Biotechnol 91:251–264
Mulligan CN, Yong RN, Gibbs BF (2001) Surfactant-enhanced remediation of contaminated soil: a review. Eng Geo 60:371–380
Osunde JE, Balogun SA (2013) Micro-organisms in enhanced oil recovery. Life J Sci 15:617–630
Owsianiak M, Chrzanowski L, Staniewski ASJ, Olszanowski A, Olejnik-Schmidt AK, Heipieper HJ (2009) Biodegradation of diesel/biodiesel blends by a consortium of hydrocarbon degraders: effect of the type of blend and the addition of biosurfactants. Bioresour Technol 100:1497–1500
Pacwa-Plociniczak M, Plaza GA, Piotrowska-Seget Z, Singh SC (2011) Environmental applications of biosurfactants: recent advances. Int J Mol Sci 12:633–654
Pacwa-Płociniczak M, Płaza GA, Poliwoda A, Piotrowska-Seget Z (2014) Characterization of hydrocarbon-degrading and biosurfactant-producing *Pseudomonas* sp. P-1 strain as a potential tool for bioremediation of petroleum-contaminated soil. Environ Sci Poll Res:1–10. doi:10.1007/s11356-014-2872-1
Rabiei A, Sharifinik M, Niazi A, Hashemi A, Ayatollahi S (2013) Core flooding tests to investigate the effects of IFT reduction and wettability alteration on oil recovery during MEOR process in an Iranian oil reservoir. Appl Microbiol Biotechnol 97:5979–5991
Rahman KSM, Rahman TJ, Kourkoutas Y, Petsas I, Marchant R, Banat IM (2003) Enhanced bioremediation of n-alkane in petroleum sludge using bacterial consortium amended with rhamnolipid and micronutrients. Bioresour Technol 90:159–168
Rikalovic M, Gojgic-Cvijovic G, Vrvic M, Karadzic I (2012) Production and characterization of rhamnolipids from *Pseudomonas aeruginosa* san-ai. J Serb Chem Soc 77:27–42
Sarachat T, Pornsunthorntawee O, Chavadej S, Rujiravanit R (2010) Purification and concentration of a rhamnolipid biosurfactant produced by *Pseudomonas aeruginosa* SP4 using foam fractionation. Bioresour Technol 101:324–330
Satpute SK, Banat IM, Dhakephalkar PK, Banpurkar AG, Chopade BA (2010) Biosurfactants, bioemulsifiers and exopolysaccharides from marine microorganisms. Biotechnol adv 28:436–450
Singh P, Cameotra SS (2004) Potential applications of microbial surfactants in biomedical sciences. Trends Biotechnol 22:142–146
Suthar H, Hingurao K, Desai A, Nerurkar A (2008) Evaluation of bioemulsifier mediated Microbial Enhanced Oil Recovery using sand pack column. J Microbiol Methods 75:225–230

Thavasi R, Sharma S, Jayalakshmi S (2011) Evaluation of screening methods for the isolation of biosurfactant producing marine bacteria. J Pet Environ Biotechnol S1:1–6. doi:10.4172/2157-7463.S1-001

Varjani SJ (2014) Hydrocarbon degrading and biosurfactants (bio-emulsifiers) producing bacteria from petroleum oil wells, PhD thesis, Kadi Sarva Vishwavidyalaya, Gandhinagar, India

Varjani SJ, Rana DP, Bateja S, Sharma MC, Upasani VN (2014) Screening and identification of biosurfactant (bioemulsifier) producing bacteria from crude oil contaminated sites of Gujarat, India. IJIRSET 3:9205–9213

Varjani SJ, Rana DP, Bateja S, Upasani VN (2013) Isolation and screening for hydrocarbon utilizing bacteria (HUB) from petroleum samples. IJCMAS 2:48–60

Varjani SJ, Rana DP, Jain AK, Bateja S, Upasani VN (2015) Synergistic ex-situ biodegradation of crude oil by halotolerant bacterial consortium of indigenous strains isolated from on shore sites of Gujarat, India. Int Biodeter Biodegr 103:116–124

Varjani SJ, Upasani VN (2012) Characterization of hydrocarbon utilizing *Pseudomonas* strains from crude oil contaminated samples. Int J Sci Computing 6:120–127

Vasconcellos SP, Dellagnezze BM, Wieland A, Klock JH, Santos Neto EV, Marsaioli AJ, Oliveira VM, Michaelis W (2011) The potential for hydrocarbon biodegradation and production of extracellular polymeric substances by aerobic bacteria isolated from a Brazilian petroleum reservoir. W J Microbiol Biotechnol 27:1513–1518

Vijaya B, Jayalakshmi NR, Manjunath K (2013) Enumeration of biosurfactant producing micro-organisms from oil contaminated soil in and around Bangalore (India). Int J Cur Sci 5:86–94

Chapter 24
Fed-Batch Cultivation for High Density Culture of *Pseudomonas* Spp. for Bioinoculant Preparation

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Abstract Fed-batch has been widely accepted in industry for commercial production of biomolecules such as enzymes, therapeutic proteins, organic acids, amino acids and other primary and secondary metabolites. Recent progress in application of fed-batch for commercial and academic research is discussed with suitable examples. This chapter deals with both the mathematical and thumb rule-based design of fed-batch cultivation. Special attention is also given to LabVIEW-based data acquisition for building control routines for implementation of customized feed operations using peristaltic pump. Finally a case study for fed-batch cultivation of *Pseudomonas* spp. is discussed.

24.1 Introduction

The seminal paper by Yamane and Shimizu (1984) is a comprehensive report on microbial cultivations using the fed-batch technique. The authors consolidated information on utility, classification, mathematical theory and survey of fed-batch cultivations. The developments in fed-batch methodology were primarily due to the problems associated with batch cultivation for improving the titers and productivities.

The depletion of essential macro elements in general and/or growth rate associated altered metabolism in facultative bacteria and consequent build-up of toxic compounds in the growth medium during batch cultivation of microbial strains' lowers the growth rate of culture eventually leading to stationary and death phases.

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If the intrinsic exhaustion of the nutritional source/s is not only monitored but also maintained in tune with the growth dynamics, the decline in growth rates can be circumvented. This external addition or feeding of rate-limiting nutrients during the growth of microbial culture forms the principle behind the fed-batch cultivation.

The primary goal of microbial cultivation is to achieve maximum possible volumetric productivity of biomass or product of interest (Riesenberg and Guthke 1999). In case of growth associated products the biomass is inherently needed to be maximized to achieve higher productivities of the product and similarly in case of non-growth associated products again the biomass is needed to be optimized to subject the cells to idiophase to produce the product of interest. Thus in either case the achievement of high cell density culture becomes a prerequisite and the fed-batch cultivation, a widely studied technique, is custom-developed to achieve this requirement. The major advantages in high cell density cultivations, apart from higher productivities, are lower reactor volumes, ease in handling downstream processing and reduced production costs. Other advantages include, prevention of growth rate dependent plasmid instability in case of production of recombinant biomolecules and also significant reduction in process times to achieve higher productivities.

This chapter essentially focusses on high cell density cultivation using fed-batch technique outlining a case study where a pseudomonas strain is subjected to various feeding strategies to improve the biomass. The chapter also elucidates the mathematical aspects for formulating the feed-profile for exponential growth and also feeding during strategy during just maintenance of the growing cultures. Moreover the chapter includes LabVIEW-based data acquisition for interfacing the peristaltic pump with the control programs for customized feeding strategies. LabVIEW code for the discussed case study is also included.

24.2 Fed-Batch, an Indispensable Mode for Microbial Cultivation

Escherichia coli and *Saccharomyces cerevisiae* are the widely used organisms among the prokaryotes and eukaryotes, respectively, for commercial exploitation. Although there are other industrially relevant organisms for production of specific compounds, these two organisms enjoy certain advantages such as well-known physiology and genetics, robust molecular techniques for modification, low doubling times, etc. At high growth rates as in batch cultures, these organisms divert carbon flux to products of anaerobic metabolism even under aerobic conditions leading to poor yield of biomass and consequent poor yield of the recombinant product. The fed-batch cultivation assumes importance as it enables the culture to grow at reduced specific growth rates to high optical densities thus obviating the problems of poor yield of biomass and the products. In fact all known feeding strategies were developed using these microbial hosts. In case of aerobic cultivation

of *E. coli*, substrate inhibition and production of ethanol, acetic and lactic acids during oxygen limitation are major issues during high cell density cultures. Especially acetic acid levels above 5 g/L is detrimental for cell cultivation, and also lowers the protein expression efficiency at low concentrations of 0.5 g/L. All the strategies were realized to contain the acetic acid production levels via lowered specific growth rates in fed-batch cultivation of *E. coli*. In case of production of recombinant proteins in *E. coli* the inducer (IPTG)—based expression system is a common strategy. Lee (1996) provides comprehensive coverage of high cell density cultivation of *E. coli* using fed-batch technique.

Zulaufverfahren was a widely accepted process where the biomass is increased without accumulation of ethanol by slowly feeding molasses during aerobic cultivation of yeast (Reed and Peppler 1973). Since then several studies have been carried out in fed-batch cultivation of *S. cerevisiae* strains. Unlike acetic acid production in case of *E. coli*, *S. cerevisiae* strains are encountered with glucose sensitivity issues during cultivation (Fiechter and Seghezzi 1992). Glucose sensitive strains produce ethanol in presence of initial glucose concentration >70 mg/L under highly aerated condition; this phenomenon is also known as Crabtree effect or overflow metabolism (Aiba et al. 1976; Pham et al. 1998). In *S. cerevisiae* fed-batch cultivation is usually carried in two stages, where in the first stage sugar is fed exponentially until oxygen limitation sets in and later shifted to the second stage where constant feed is supplied to achieve quasi-steady state with respect to substrate and concomitant lowering of specific growth rate (Pham et al. 1998). The biomass formation is dependent on glucose consumption by growing cells and the fresh substrate addition is controlled during first stage precisely to overcome overflow metabolism.

Although these strategies were developed 20–25 years ago, they have been continuously evolved to suit either for newly developed strains or for novel products being produced. Some of the developments in fed-batch cultivation of these two industrial workhorses are provided in Table 24.1.

Hence, fed-batch technique has become an indispensable tool for both academic research and commercial production of biomolecules.

24.2.1 Types of Fed-Batch Cultivation

The classification of the fed-batch operation are usually based on understanding two key actions, viz., (1) how the feeding is carried out and (2) what is responsible for such feeding action. All feeding strategies are connected to physiological state of the growing culture and with prime motive to avoid the very problem of under feeding or over feeding of limiting nutrients (Lee et al. 1999). The fed-batch cultivation can be broadly classified into open-loop (without feedback) and closed-loop (with feedback) modes according to Yamane and Shimizu (1984).

Table 24.1 Some of the prominent fed-batch strategies for *Escherichia coli* and *Saccharomyces cerevisiae*

Organism	Fed-batch strategy	Desired result	References
Escherichia coli	Glucose was fed based on inference of specific growth rate (μ) from off-gas data. μ was maintained at 0.11 h^{-1} to achieve 95 g/L	Correlation between specific growth rate and oxygen uptake rate	Riesenberg et al. (1991)
	The specific growth rate (μ) was controlled to minimize acetic acid production using exponential feeding profile. $\mu < 0.2\ h^{-1}$, to achieve 92 g/L	Determination of exponential feed profile using mass balance	Yee and Blanch (1992)
	Exponential feeding with pre-determined specific growth rate (μ) < 0.17 h^{-1} to achieve 128 g/L and 148 g/L of biomass with glucose and glycerol, respectively as carbon sources	Incorporation of volume correction in feed profile estimation due to sampling and addition of other limiting nutrients	Korz et al. (1995)
	The concentration of glucose inside the reactor was maintained at 1.5 g/L by continuous measurement using flow injection device and correspondingly regulating the feed rate	FIA-based fed-batch cultivation to achieve high cell density of 145 g/L	Horn et al. (1996)
	IPTG limitation during induction phase lowers the acetic acid production during induction phase thereby improving the enzyme yields in fed-batch cultivation	The study demonstrates that feeding during glucose limitation improves high cell density and feeding with IPTG limitation reduces acetic acid formation	Lecina et al. (2013)
Saccharomyces cerevisiae	Step-wise feeding of glucose such that the respiratory quotient (RQ) ranged between 1.0 and 1.2 during fed-batch	*RQ*, was estimated by calculating the difference of the partial pressures of O_2 and CO_2 in air between air inlet and exit gas	Aiba et al. (1976)
	Inference of maximum substrate uptake rate (MSUR) by on-line measurements of dissolved oxygen (DO) and thereby controlling the feeding for aerobic *S. cerevisiae* cultures	This strategy improved the production of recombinant gluco-amylase significantly	Oh et al. (1998)
	Respiratory quotient (RQ) was maintained between 4 and 5 during anaerobic fed-batch cultivation to improve ethanol production	Reduced the surplus formation of NADH which is usually responsible for glycerol formation as a byproduct along with ethanol	Bideaux et al. (2006)

(continued)

Table 24.1 (continued)

Organism	Fed-batch strategy	Desired result	References
	Model-driven dynamic optimization of fed-batch feeding policies to maximize ethanol by constraining with flux balances, extracellular balances and standard kinetics	Mass balances on extracellular species are coupled to a stoichiometric model of intracellular metabolism through substrate uptake kinetics and product secretion rates	Hjersted and Henson (2006)
	After depletion of ethanol from batch phase the lignocellulose hydrolysate was fed linearly between 0.04 and 0.1 L/h over 16 h to achieve maximum biomass	This strategy improved productivity of *S. cerevisiae* in the presence of inhibitors in feed and also making them adapted for further processing	Olofsson et al. (2008)

In case of open-loop strategies, the feeding of the limiting substrate is carried out without considering the outcome of fed-batch operation, however it is based on heuristics or a priori knowledge. In case of closed-loop fed-batch cultivation the feed manipulation is inferred from measurement of a state variable which changes during fed-batch cultivation (Lee 1996). The different types of fed-batch cultivations are shown diagrammatically in Fig. 24.1. The Fig. 24.1a depicts the time profiles of some of the standard open-loop feeding strategies. Here the feeding is simply based on prior knowledge of the physiology of the culture without consideration of the dynamics of culture during feeding. This includes constant, linear, step-wise or pulse feeding and exponential feeding.

In case of Fig. 24.1b feed profiles of closed-loop strategies have been depicted with indirect measurement variables such as dissolved oxygen (DO), pH and respiratory quotient (RQ). These variables are widely used to understand the physiology of the growing culture as they are sensitive to depletion of limiting nutrients in the culture broth. In case of DO, the value rises sharply after depletion of main carbon/energy source and this is when feed is turned-on after it goes above a set value (DO_set_value, cf. Fig. 24.1b), and the feed is turned-off below a critical value (DO_critical_value) when oxygen supply becomes limiting. During the period between on-off times, the culture is allowed to grow. For pH-based feeding, the culture pH increases once the carbon source is depleted due to release of ammonium ions. As the pH rises above a user-set upper value (pH_Uset_value), two simultaneous actions takes place: (1) the addition of ammonium solution to controlthepH is turned-off and (2) the feed pump is turned-on for a specific time period (t_{on}, cf. Fig. 24.1b); and afterwards due to consumption of added fresh substrate, the pH starts falling sharply and as it falls below a user set lower value (pH_Lset_value) the ammonium pump is switched-on (Suzuki et al. 1990; Sun et al. 2006). In case of RQ-based feeding an RQ value of 1.0 signifies that the rate of consumption of oxygen is equal to CO_2 evolution rate. As the value falls, it

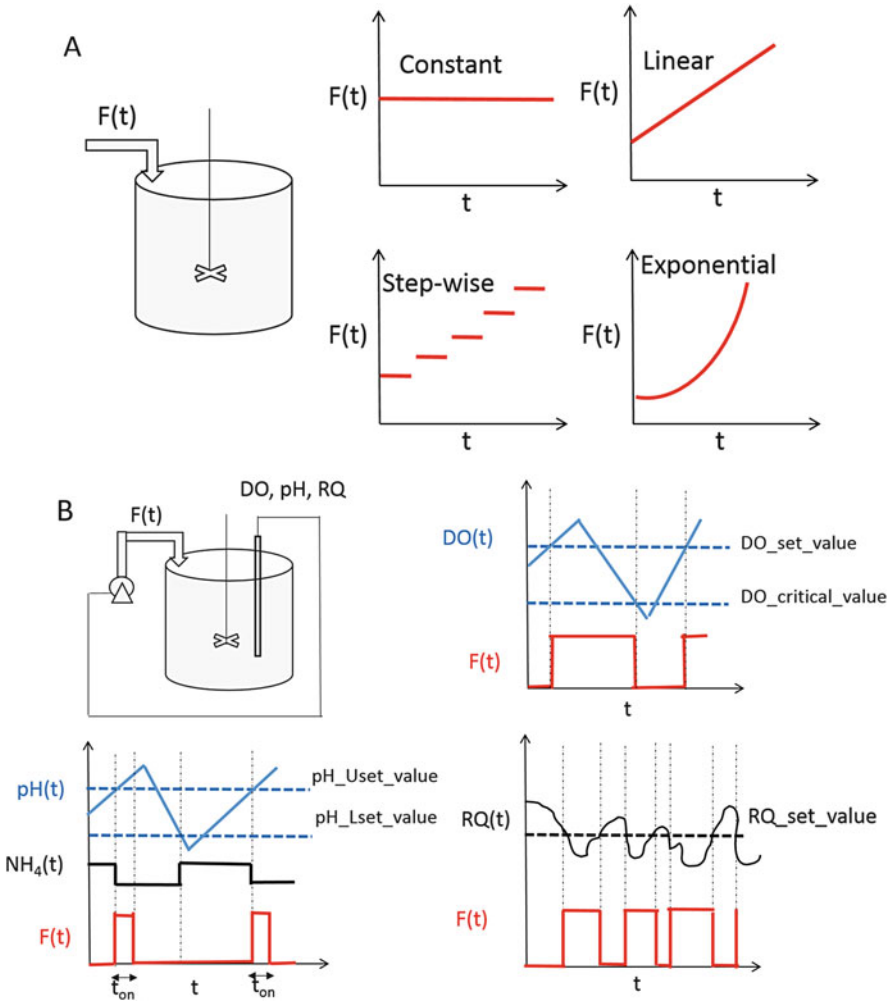

Fig. 24.1 Depiction of (**a**) open-loop feeding strategies (**b**) closed-loop feeding strategies

indicates the depletion of the limiting substrate and hence the feed-pump is switched-on; and thereafter it is switches-off when the RQ goes above a set value (RQ_set_value). Table 24.2 provides information on few recent fed-batch strategies employed for production of bioproducts.

Table 24.2 A few recent fed-batch strategies used for production of bioproducts

Fed-batch strategy (using rate-limiting substrate for feeding)	Microorganism	Product(s)	References
Exponential feeding of galactose at $\mu = 0.05\ h^{-1}$	Engineered *E. coli*	Lacto-*N*-tetraose (12.72 g/L) Lacto-*N*-triose (13.70 g/L)	Baumgärtner et al. (2015)
Glucose (500 g/L) was fed at a constant rate of 7 mL/h during 48–72 h and 4.67 mL/h during 72–108 h	*Mortierella alpine* (mutant)	Arachidonic acid 8.37 g/L	Li et al. (2015a)
Exponential feeding of glucose (400 g/L) with fixed specific growth rate of $0.2\ h^{-1}$	Engineered *E. coli*	Valinomycin 2 mg/L	Li et al. (2015b)
Exponential feeding of glycerol with fixed specific growth rate of $0.2\ h^{-1}$	Engineered *E. coli*	4-hydroxybutyrate 15 g/L	Le Meur et al. (2014)
Exponential feeding of butyric acid with fixed specific growth rate of $0.2\ h^{-1}$	*Pseudomonas putida*	DCW 71.3 g/L	Cerrone et al. (2014)
Exponential feeding of commercial palm oil with specific growth rate of $0.05\ h^{-1}$	*Candida rugosa* ATCC 10571	Lipase 699 U/L	Salehmin et al. (2014)
Two stage pH-stat fed batch Stage1: 500 g/L fructose with 150 g/L NH_4Cl Stage2: 500 g/L fructose with 12.5 g/L NH_4Cl	Engineered *R. eutropha*	Branched-chain alcohol 0.79 g/L	Fei et al. (2013)
DO-stat feeding of glycerol Feeding was paused if DO $<$ 20 % and continued when the DO $>$ set value	Engineered *P. pastoris*	Ice binding protein 272 mg/L	Lee et al. (2013)

24.3 Mathematical Model-Based Feeding Profiles for Fed-Batch Cultivation

The fed-batch phase commences after batch growth ceases on account of exhaustion of limiting nutrients (Fig. 24.2). During fed-batch phase, highly concentrated limiting nutrients are supplied in small doses in a specific manner and the volume of the reactor increases.

The nomenclatures of the terms shown in Fig. 24.1 are as follows:

x_0 Initial biomass concentration just after inoculation, g/L
x biomass concentration at any instance of time, g/L
x_i biomass concentration at the end of batch phase, g/L
x_f biomass concentration at the end of fed-batch phase, g/L
V_i volume of reactor in batch phase, L
V volume of reactor in fed-batch phase at any instance of time, L

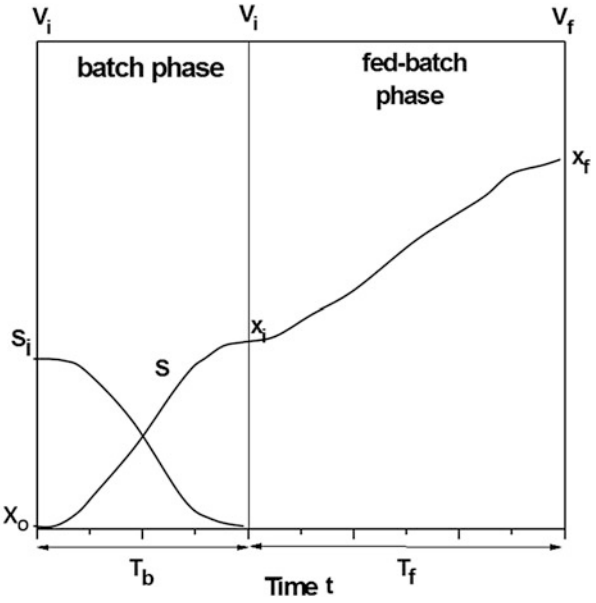

Fig. 24.2 Representation of fed-batch cycle with state variable profiles along time

V_f volume of reactor at the end of fed-batch phase, L
S_0 substrate concentration in feed, g/L
S substrate concentration at any time in fed-batch phase, g/L
t time, h
T_b batch time, h
T_f fed-batch time, h
μ specific growth rate (h^{-1})

The model equations governing growth kinetics during fed-batch phase are provided below:

Feed rate (L/h) equation

$$F = \frac{dV}{dt} \tag{24.1}$$

Specific growth rate (μ, h^{-1})

$$\mu = \frac{1}{xV}\frac{d(xV)}{dt} \tag{24.2}$$

$$xV = x_i V_i e^{\mu t} \tag{24.3}$$

Substrate balance for fed-batch phase

Accumulation = input – output – consumption

$$\frac{\mathrm{d}(SV)}{\mathrm{d}t} = FS_0 - \frac{\mu xV}{Y_{x/s}} - mxV \tag{24.4}$$

Where, m = maintenance co-efficient, h^{-1}
$Y_{x/s}$ = biomass yield with respect to substrate consumed, g/g
Integration of substrate balance Eq. (24.4) gives following equation

$$V\frac{\mathrm{d}S}{\mathrm{d}t} + S\frac{\mathrm{d}V}{\mathrm{d}t} = FS_0 - \left(\frac{\mu x_i V_i e^{\mu t}}{Y_{x/s}} + mxV\right) \tag{24.5}$$

Assuming quasi-steady state with respect to limiting substrate (i.e. S does not change with time), the following equation is achieved

$$FS = FS_0 - \left(\frac{\mu x_i V_i e^{\mu t}}{Y_{x/s}} + mxV\right) \tag{24.6}$$

$$F(S_o - S) = \left(\frac{\mu}{Y_{x/s}} + m\right) x_i V_i e^{\mu t} \tag{24.7}$$

24.3.1 A priori Models for Fed-Batch

Exponential feeding falls in the category a priori fed-batch system, where the specific growth is fixed at a certain value and it is expected that the culture grows exponentially at this fixed value. Usually, values below 0.2 h^{-1} are preferred as at higher values the growing culture may not adapt to incoming dosage of fresh feed and may eventually lead to substrate inhibition due to accumulation of the limiting substrate. However the μ value and concentration of feed is heuristically decided based on the physiology of the culture from batch experiments.

From Eq. (24.7)

$$F = \frac{\left(\frac{\mu}{Y_{x/s}} + m\right) x_i V_i e^{\mu t}}{(S_o - S)} \tag{24.8}$$

As the concentration of the fresh nutrient is very high in comparison to the residual concentration at the end of batch phase, the following case is assumed:

$$S_o >> S \Rightarrow S_o - S = S_o$$

$$F = \frac{\left(\frac{\mu}{Y_{x/s}} + m\right) x_i V_i e^{\mu t}}{S_o} \tag{24.9}$$

If $\alpha = \frac{\left(\frac{\mu}{Y_{x/s}} + m\right) x_i V_i}{S_o}$ and

$$\beta = \mu$$
$$F(t) = \alpha e^{\beta t} \tag{24.10}$$

The Eq. (24.10) is used for exponential feeding to grow the culture at a desired constant specific growth rate. Here in right side of the Eq. (24.10) all the values are known (in case of maintenance co-efficient, the value is either calculated from batch/continuous culture experiments or assumed based on literature information for the culture) and the feed profile is thus determined for the fed-batch cultivation to be implemented.

In some cases the maintenance co-efficient is assumed to be negligible hence Eq. (24.10) becomes

$$F(t) = \frac{\mu x_i V_i e^{\mu t}}{Y_{x/s} S_o} \tag{24.11}$$

However, maintenance term must be considered particularly in cases of recombinant cultures where the growth rate is significantly reduced during induction phase.

In case of maintenance phase, growth rate is assumed negligible, i.e. $\mu = 0$. Hence, the feed rate in Eq. (24.11) assumes following equation:

$$F = \frac{m x_i V_i}{S_o} \tag{24.12}$$

This equation implies that constant flow rate should be maintained for meeting the maintenance requirements by feeding in stationary phase. For finding the initial flow-rate for predetermined feeding (i.e., at constant μ) the following calculations can be carried out.

$$\int_{V_0}^{V_t} \mathrm{d}V = \int_{t=0}^{t} F(t)\mathrm{d}t$$

$$V_t - V_0 = \int_{t=0}^{t} \alpha e^{\beta t}\mathrm{d}t$$

Where, $\alpha = \frac{\left(\frac{\mu}{Y_{x/s}} + m\right) x_i . V_i}{S_o}$ and $\beta = \mu$

$$V_t = V_0 + \frac{\alpha}{\beta}\left(e^{\beta t} - 1\right) \tag{24.13}$$

24.3.2 Optimal Control of Fed-Batch Cultivation

A continuous dynamic system is described by following differential equation (Luus 2000)

$$\frac{\mathrm{d}\mathbf{x}}{\mathrm{d}t} = f(\mathbf{x}, \mathbf{u}, t) \quad \text{or}$$
$$\frac{\mathrm{d}\mathbf{x}}{\mathrm{d}t} = \mathbf{A}\mathbf{x} + \mathbf{B}\mathbf{u} \tag{24.14}$$

x is an $(n \times 1)$ state vector and u is an $(m \times 1)$ control vector bounded by

$$\alpha_j \leq u_j(t) \leq \beta_j, \quad j = 1, 2, ..., m$$

The performance index is chosen as a scalar function and defined as below

$$I\left[\mathbf{x}(0), t_f\right] = \psi\left(\mathbf{x}\left(t_f\right)\right) + \int_0^{t_f} \phi\left(\mathbf{x}, \mathbf{u}, t\right)\mathrm{d}t \tag{24.15}$$

Here $x(0)$ initial state and final time t_f are specified and the optimal control policy $u(t)$ in the time interval [0, t_f] is selected such as the performance index is maximized. The other variations of optimal control problems include constraints for the state variables and time optimal control problems.

Dynamic equations for fed-batch fermentation are follows:

$$\frac{\mathrm{d}(xV)}{\mathrm{d}t} = \mu xV; x(0) = x_0$$
$$\frac{\mathrm{d}(SV)}{\mathrm{d}t} = FS_F - \frac{\mu xV}{Y_{x/s}} - mxV; s(0) = s_0$$

$\frac{\mathrm{d}(pV)}{\mathrm{d}t} = q_p xV; p(0) = p_0$; Where q_p is specific product formation rate

Table 24.3 Different techniques to solve the optimal feeding profile in fed-batch cultivation

Technique	References
Optimal control theory using multi-point boundary value problem	Lim et al. (1986) Park and Ramirez (1988) Modak and Lim (1989)
Discretization of time into sub-intervals and constant feed during those sub-intervals	Shukla and Pushpavanam (1998) (Sequential quadratic programming, SQP) Banga et al. (1998) (Integrated Controlled Random Search for Dynamic Systems, ICRS/DS) Luus (1994) (Iterative dynamic programming, IDP)
Genetic algorithms	Sarkar and Modak (2003)
Simulated annealing	Kookos (2004)
Differential evolution	Rocha et al. (2014)
Particle swarm optimization	Jones (2006), Liu et al. (2009), Rocha et al. (2014)

$$\frac{\mathrm{d}V}{\mathrm{d}t} = F; V(0) = V_0$$

Constraints for the process are as follows:

$$V(t_f) = V_f$$
$$0 = F_{\min} \leq F(t) \leq F_{\max}$$

$$\frac{\mathrm{d}}{\mathrm{d}t}\begin{bmatrix} x_1 \\ x_2 \\ x_3 \\ x_4 \end{bmatrix} = \begin{bmatrix} \mu x_1 \\ -\dfrac{\mu x_1}{Y_{x/s}} - m x_1 \\ q_p x_1 \\ 0 \end{bmatrix} + \begin{bmatrix} 0 \\ S_F \\ 0 \\ 1 \end{bmatrix} F$$

Here $x_1 = xV$, $x_2 = sV$, $x_3 = pV$ and $x_4 = V$

For instance if product or metabolite p has to be maximized the problem would be as follows

$$\underset{F(t)}{\text{Maximize}}\ PI = (x_3)_{t_f}$$
subject to
$$V(t) \leq V_{\max}$$
$$0 = F_{\min} \leq F(t) \leq F_{\max}$$
$$0 \leq t \leq t_f$$

The solution for above optimal control problem has been evaluated by several techniques and is consolidated in Table 24.3.

24.4 LabVIEW-Based Data Acquisition and Control for Fed-Batch Cultivation

Biochemical processes are difficult to control due to their non-linear nature. The sensitivity of the microorganisms and the inability to fully influence the internal environment of cells by manipulating the external environment in which they live has been a challenge in bioprocess control (Rani and Rao 1999). The built-in software of most of the commercial bioreactors can display the process values (pH, rpm, temperature and DO), but its capabilities for feed-control, based on process values such as DO and pH, and driving the pump at exponential rate for predetermined feed-strategy are limited (Huang et al. 2005). Hence there is a requirement for design of custom control mechanisms for carrying out fed-batch fermentations.

LabVIEW is an interactive graphical programming tool for automation of processes from National Instruments, Inc. (Austin, TX, USA). It has extensive libraries of standard functions and subroutines, and specialized libraries for data acquisition (DAQ). The program is developed as a virtual instrument (VI) which contains front panel and block diagram. Front panel of a VI is the interactive part of the code where the inputs for the test code are provided and also the outputs can be viewed as graphs and other indicators in real times. The block diagram is the VIs actual executable code. It contains built-in functions, constants, and program execution control structures connecting wires between the objects for proper flow of the information (Travis and Kring 2006).

24.4.1 Serial-Port Communication with the Feed Pump

The connection between the bioreactor and the computer running LabVIEW 'VI' is usually established through a non-powered RS-232, RS-422 or RS-485 connected to the computer's serial port**.** Nowadays the USB-Serial adapters are popular for establishing connections in case serial port is not available on the workstation.RS-232 is the simple and most widely used serial port among the three. It can work in full-duplex, implying the information can be received as well as sent using this serial port (Bai 2004). A multifunctional PCI card is needed to be fitted into the serial slot of the workstation. A PCL shielded cable is required to connect the PCI card and the DIN rail wiring board, which in turn provides the voltage signal (0–10 V) for operating any motor drive (a peristaltic pump in case of fed-batch fermentation). It also has digital inputs and outputs which are used to monitor status of limit switches and opening or closing of solenoid valves respectively. The connection of the physical components is pictorially shown in Fig. 24.3.

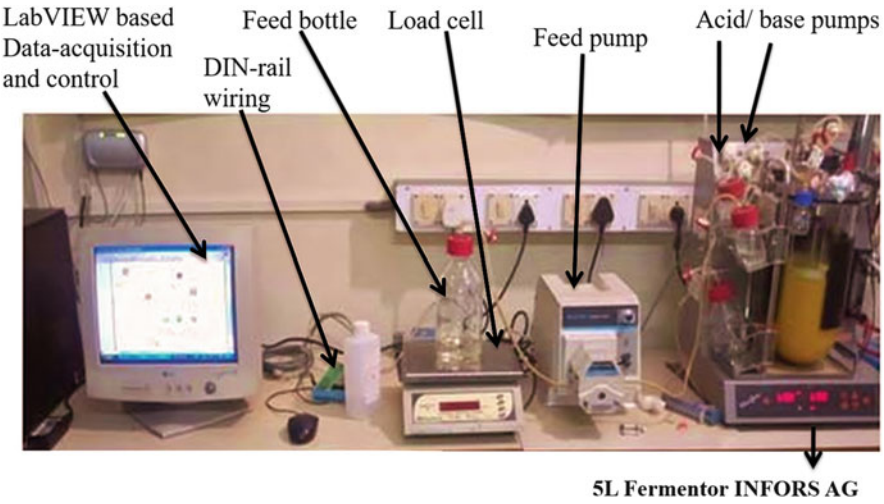

Fig. 24.3 Set-up for fed-batch cultivation of fluorescent pseudomonad R81

Table 24.4 COM port choices for establishing serial connection

Baude rate	110, 300, 600, 1200, 2400, 4800, 9600 or 19,200
Data bits	4, 5, 6, 7 or 8
Parity	Odd, None, Mark or Space
Stop bits	1, 1.5 or 2

24.4.2 Data Acquisition of Bioreactor Variables onto LabVIEW

The online variables measured using the sensors (temperature, pH, RPM, dissolved oxygen, $\%CO_2$ and $\%O_2$ in exit gas) are invariably displayed or indicated either on the bioreactor or on the accompanying software. In order to bring the same information on to the customized VI of LabVIEW, a serial port connection is established between the remote COM port of the bioreactor and the workstation. Later the port is invoked using LabVIEW's basic read–write VI module with COM port settings of the bioreactor. The common setting options to choose are shown in Table 24.4.

The port settings for serial communication for Minifors, Infors AG, Switzerland are as follows: SPEED = 9600 bd, DATA- = 8 bit, STOP = 1 bit, PARTY = None, FLOW CONTROL = None. Once the data string is read onto the VI, further changes are brought in the block diagram to modify the outputs as per the requirement. Sometimes a request has to be sent through a string of code to receive the digital data from bioreactor. The request code was of the following type:

Request code: "\XXXX;R\s\sA\XX"

This code is usually provided by the supplier and varies with the model. Once *reading* is established, the next task is to send signals (or *writing*) to the bioreactor for any changes in the inputs or set-values. Here again the request needs to be sent as a string code as specified by the supplier. Once the read–write module is established onto a VI, the pump voltage is wired with the desired feed-back variable (such as pH, DO or RQ) or in case of predetermined exponential feeding a mathematical subroutine for exponential function can be utilized to invoke the external pump.

24.5 Case Study for High Cell Density Cultivation of a Pseudomonad Strain

Fluorescent pseudomonad R81 is an established plant growth promoting rhizobacterium (Gaur et al. 2004) and application of its formulation to wheat crop in field studies has been shown to significantly improve the crop growth parameters (Mäder et al. 2011). It has been observed in our earlier study (Saharan et al. 2011) that the antibiotic 2,4-diacetylphloroglucinol (DAPG) produced by this strain in the culture broth (which is used for preparing powder formulation) essentially keeps the contaminants under check when the formulated powder is under storage. Moreover, the presence of such biocontrol agent as the active ingredient of the bioinoculant formulation would initiate the antagonistic action against the soil-borne pathogens when the formulation is applied to the rhizosphere. Furthermore, high colony forming units (CFU/mL) of the bioinoculant in the culture broth will enable us to prepare its formulation with desired CFU per gram formulation by using lesser volumes of the culture broth. Thus, the aim of the fermentation process was to cultivate the strain in high cell density in order to prepare an effective bioinoculant formulation. The high cell density cultures along with synthesis of DAPG would significantly improve both process efficiency and product efficacy. To achieve high cell density cultures, a synthetic medium with higher dosage of glycerol was used to conduct batch experiments (Sarma et al. 2013). The strain R81 utilizes glycerol as the principal carbon and energy source for its growth; however under high initial glycerol concentration ($\geq$15 g/L) the specific growth rate falls significantly due to substrate inhibition (Sarma et al. 2013). Hence fed-batch cultivation of the strain R81 was planned as a potential mode of cultivation to achieve high cell density. Several fed-batch cultivation strategies employing *Pseudomonas* spp. have been reported; for instance Sun et al. (2006) used exponential feeding of glucose for improving biomass using *Pseudomonas putida*KT2440, Kim (2002) adopted pH-based feeding of octanoic acid for the strain *Pseudomonas oleovorans* ATCC 29347 in order to increase polyhydroxy alkanoates production and Suzuki et al. (1988) fed olive oil at constant rate during cultivation of *Pseudomonas fluorescens* strain to produce lipase. However, these studies focused on a specific product not related to a bioinoculant.

Hence both open-loop (dosing the substrate exponentially) and closed-loop feeding strategies (DO- and pH-based) were employed to achieve high cell density of R81 in fed-batch cultivation (Sarma et al. 2013). It was observed that exponential feeding with the fixed specific growth rate of 0.10 and 0.20 h^{-1} failed due to accumulation of glycerol and thereby triggering substrate inhibition. Hence closed-loop strategies such as dissolved oxygen (DO) and pH were designed in order to indirectly understand the physiology and residual sugar present in the bioreactor during fed-batch cultivation of strain R81.Here the basic principle remains similar to that of Suzuki et al. (1990) as depicted in Fig. 24.1b with certain modifications. In case of DO-based fed-batch, the working principle is based on the logic that whenever the limiting carbon is exhausted, the DO signal shoots up instantaneously and settles at the saturation level as there is no active oxygen uptake by the cells. Similarly when the limiting substrate is fed, there would be a gradual drop in the DO signal as the cells present in the bioreactor will start taking up the dissolved oxygen via respiration. Instead of directly using DO signal for feeding, a modified algorithm was designed by considering the slope of DO signal and the sign of this slope was used to decide whether the pump has to be switched-on or not. If the slope was positive, the pump was switched-on and if it was negative, the pump was turned-off automatically using the LabVIEW interface to drive the pump. This strategy resulted in accumulation of 25 g/L of biomass along with 250 mg/L of DAPG at the end of 72 h of cultivation (Sarma et al. 2013).

In the case of pH-based fed-batch technique, the working principle was based on the fact that pH rises once the limiting carbon is exhausted due to release of ammonium ions by cells and similarly during consumption of the substrate, the pH usually drops (Yamane and Shimizu 1984). The major change was that the feeding was carried out only within the dead band of the pH controller of Minifors bioreactor. The chosen controller set value for pH was 6.90 with a dead band of 6.90 ± 0.05 as shown in the Fig. 24.4.

Hence within this dead band, the pH control action will not take place and the ammonia pump will remain switched off. Here within this dead band, a user set value of 6.92 was provided and as the pH rose above this value, the glycerol pump was switched-on again for calculated t_{on} time such that the glycerol concentration

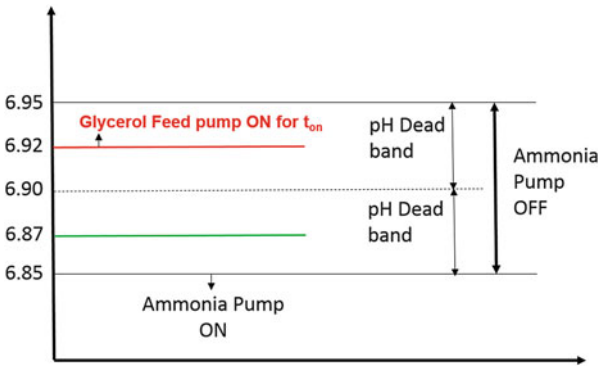

Fig. 24.4 Feeding strategy for pH-based fed-batch cultivation of fluorescent pseudomonad R81

increased to 2 g/L in the reactor. The ammonia pump was turned on whenever the pH was below 6.85 and it never rose above 6.87 as the cells were actively growing. Thus the pH 6.92 was selected as user defined value to ensure that the pH rise was due to exhaustion of glycerol and not due to addition of ammonia solution (Sarma et al. 2013). The feeding was based on the logic as depicted in Fig. 24.4. Using pH-based cultivation, maximum biomass of 27 g/L along with 342 mg/L of DAPG were achieved at the end of 50 h of cultivation, thereby increasing the productivity of biomass in comparison to DO-based feeding (Sarma et al. 2013). The time course data for strain R81 during fed-batch cultivation are shown in Fig. 24.5 (Sarma et al. (2013). The same pH signal-based feeding strategy was also applied for high density cultivation of another *Pseudomonas* sp. (strain R62) and similar enhancements in biomass productivity were observed (Sarma et al. 2013). Thus pH signal-based feeding strategy was observed to be highly robust for high cell density cultivation of the fluorescent pseudomonads with highest biomass productivity.

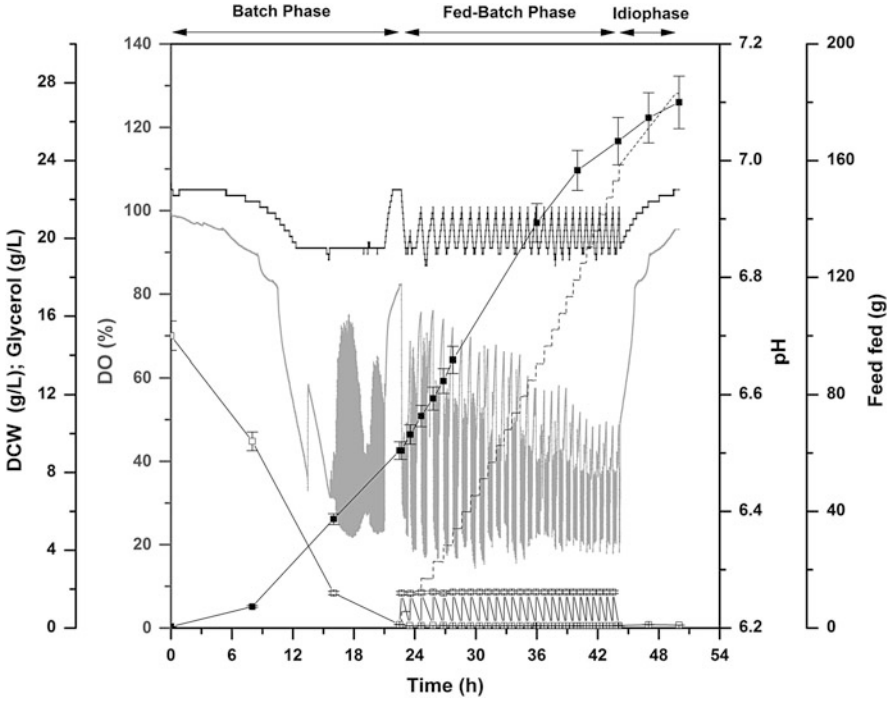

Fig. 24.5 Time course of the pH-signal based fed-batch cultivation of fluorescent pseudomonad R81 in a 5 L bioreactor. The feeding commenced at 22.5 h after batch cultivation and continued up to 44 h followed by constant feeding for 6 h up to 50 h. *Solid grey line* denotes dissolved oxygen (% saturation), the *solid black line* denotes pH profile. *Filled square* denotes dry cell weight (g/L) and *hollow square* denotes glycerol (g/L) profile. Total feed medium fed into the bioreactor is shown by *stepped line* (Sarma et al. 2013)

Therefore from process point of view it has been established that fed-batch mode of cultivation for potential bioinoculant strains of *Pseudomonas* spp. could improve both the process efficiency and product efficacy. Commercial formulations of *Pseudomonas*-based bioinoculants are either marketed as phosphate solubilizers or biocontrol agents against plant-borne pathogens. Also certain bioinoculant formulations include *Pseudomonas* spp. in their consortia in order to broaden the efficacy of the product. Some of these include Blight Ban A506 (Nufarm, Australia), Bactvipe (International Panacea Ltd., India), BactoFil A10 (AgroBio, Hungary) etc. Hence,the fed-batch cultivation strategies can be effectively used for high cell density cultures of the*Pseudomonas*—based bioinoculants.

24.6 Conclusions

Fed-batch mode of operation of a bioreactor results in improved productivity of biomass and biomolecules during microbial cell cultivation, and hence it is an indispensable tool for industrial production of bioproducts. In this chapter, various types of fed-batch cultivation strategies were discussed based on understanding of a cell's physiological condition, and event-based driving of the feed pump. Standard mathematical calculations can be used to design exponential and optimal feeding in which the feed-profile is designed as a priori. The application of fed-batch modes for high cell density cultivation of a potential bio-inoculant strain belonging to *Pseudomonas* spp., exemplified as a case study, can be employed gainfully for other bio-inoulants.

References

Aiba S, Nagai S, Nishi Y (1976) Fed batch culture of *Saccharomyces cerevisiae*: a perspective of computer control to enhance the productivity in baker's yeast cultivation. Biotechnol Bioeng 18:1001–1016

Bai Y (2004) The windows serial port programming handbook. Auerbach Publications/ACRC Press, New York

Banga JR, Irizarry-Rivera R, Seider WD (1998) Stochastic optimization for optimal and model-predictive control. Comput Chem Eng 22:603–612

Baumgärtner F, Sprenger GA, Albermann C (2015) Galactose-limited fed-batch cultivation of *Escherichia coli* for the production of lacto-N-tetraose. Enzym Microb Technol 75–76:37–43

Bideaux C, Alfenore S, Cameleyre X, Molina-Jouve C, Uribelarrea JL, Guillouet SE (2006) Minimization of glycerol production during the high-performance fed-batch ethanolic fermentation process in *Saccharomyces cerevisiae*, using a metabolic model as a prediction tool. Appl Environ Microbiol 72:2134–2140

Cerrone F, Duane G, Casey E, Davis R, Belton I, Kenny ST, O'Connor K (2014) Fed-batch strategies using butyrate for high cell density cultivation of *Pseudomonas putida* and its use as a biocatalyst. Appl Microbiol Biotechnol 98:9217–9228

Fei Q, Brigham CJ, Lu J, Fu R, Sinskey AJ (2013) Production of branched-chain alcohols by recombinant *Ralstonia eutropha* in fed-batch cultivation. Biomass Bioenergy 56:334–341

Fiechter A, Seghezzi W (1992) Regulation of glucose metabolism in growing yeast cells. J Biotechnol 27:27–45

Gaur RS, Noam S, Kawaljeet K, Johri BN, Rossi P, Aragno M (2004) Diacetylphloroglucinol producing pseudomonads do not influence AM fungi in wheat rhizosphere. Curr Sci 86:453–457

Hjersted JL, Henson MA (2006) Optimization of fed-batch *Saccharomyces cerevisiae* fermentation using dynamic flux balance models. Biotechnol Prog 22:1239–1248

Horn U, Strittmatter W, Krebber A, Knüpfer U, Kujau M, Wenderoth R, Riesenberg D (1996) High volumetric yields of functional dimeric miniantibodies in *Escherichia coli*, using an optimized expression vector and high-cell-density fermentation under non-limited growth conditions. Appl Microbiol Biotechnol 46:524–532

Huang H, Gu T, Moo-Young M (2005) Data acquisition and control of a 22 L B. Braun fermenter using LabVIEW. Chem Eng Commun 192:137–144

Jones KO (2006) Comparison of genetic algorithms and particle swarm optimisation for fermentation feed profile determination. In: International conference on computer systems and technologies – Comp Syst Tech' 2006

Kim BS (2002) Production of medium chain length polyhydroxyalkanoates by fed-batch culture of *Pseudomonas oleovorans*. Biotechnol Lett 24:125–130

Kookos IK (2004) Optimization of batch and fed-batch bioreactors using simulated annealing. Biotechnol Prog 20:1285–1288

Korz DJ, Rinas U, Hellmuth K, Sanders EA, Deckwer WD (1995) Simple fed-batch technique for high cell density cultivation of *Escherichia coli*. J Biotechnol 39:59–65

Le Meur S, Zinn M, Egli T, Thöny-Meyer L, Ren Q (2014) Improved productivity of poly (4-hydroxybutyrate) (P4HB) in recombinant *Escherichia coli* using glycerol as the growth substrate with fed-batch culture. Microb Cell Factories 13:131. doi:10.1186/s12934-014-0131-2

Lecina M, Sarró E, Casablancas A, Gòdia F, Cairó JJ (2013) IPTG limitation avoids metabolic burden and acetic acid accumulation in induced fed-batch cultures of *Escherichia coli* M15 under glucose limiting conditions. Biochem Eng J 70:78–83

Lee SY (1996) High cell-density culture of *Escherichia coli*. Trends Biotechnol 14:98–105

Lee J, Lee SY, Park S, Middelberg APJ (1999) Control of fed-batch fermentations. Biotechnol Adv 17:29–48

Lee JH, Lee SG, Do H, Park JC, Kim E, Choe YH, Kim HJ (2013) Optimization of the pilot-scale production of an ice-binding protein by fed-batch culture of *Pichiapastoris*. Appl Microbiol Biotechnol 97:3383–3393

Li X, Lin Y, Chang M, Jin Q, Wang X (2015a) Efficient production of arachidonic acid by *Mortierella alpina* through integrating fed-batch culture with a two-stage pH control strategy. Bioresour Technol 181:275–282

Li J, Jaitzig J, Lu P, Süssmuth RD, Neubauer P (2015b) Scale-up bioprocess development for production of the antibiotic valinomycin in *Escherichia coli* based on consistent fed-batch cultivations. Microb Cell Factories 14:83. doi:10.1186/s12934-015-0272-y

Lim HC, Tayeb YJ, Modak JM, Bonte P (1986) Computational algorithms for optimal feed rates for a class of fed-batch fermentation: numerical results for penicillin and cell mass production. Biotechnol Bioeng 28:1408–1420

Liu C, Gong Z, Feng E, Yin H (2009) Modelling and optimal control for nonlinear multistage dynamical system of microbial fed-batch culture. J Indus Manage Opt 5:835–850

Luus R (1994) Optimal control of batch reactors by iterative dynamic programming. J Process Control 4:218–226

Luus R (2000) Iterative dynamic programming. Chapman & Hall/CRC Press, Boca Raton

Mäder P, Kaiser F, Adholeya A, Singh R, Uppal HS, Sharma AK, Srivastava R, Sahai V, Aragno M, Wiemken A, Johri BN, Fried PM (2011) Inoculation of root microorganisms for sustainable wheat–rice and wheat–black gram rotations in India. Soil Biol Biochem 43:609–619

Modak JM, Lim HC (1989) Simple nonsingular control approach to fed-batch fermentation optimization. Biotechnol Bioeng 33:11–15

Oh G, Moo-Young M, Chisti Y (1998) Automated fed-batch culture of recombinant *Saccharomyces cerevisiae* based on on-line monitored maximum substrate uptake rate. Biochem Eng J 1:211–217

Olofsson K, Rudolf A, Lidén G (2008) Designing simultaneous saccharification and fermentation for improved xylose conversion by a recombinant strain of *Saccharomyces cerevisiae*. J Biotechnol 134:112–120

Park S, Ramirez WF (1988) Optimal production of secreted protein in fed-batch reactors. AIChE J 34:1550–1558

Pham HTB, Larsson G, Enfors SO (1998) Growth and energy metabolism in aerobic fed-batch cultures of *Saccharomyces cerevisiae*: Simulation and model verification. Biotechnol Bioeng 60:474–482

Rani KY, Rao VSR (1999) Control of fermenters – a review. Bioprocess Eng 21:77–88

Reed G, Peppler HJ (1973) Baker's yeast production. In: Reed G, Peppler HJ (eds) Yeast technology. Avi Publishing, Westport, pp 53–102

Riesenberg D, Schulz V, Knorre WA, Pohl HD, Korz D, Sanders EA, Ross A, Deckwer WD (1991) High cell density cultivation of Escherichia coli at controlled specific growth rate. J Biotechnol 20:17–27

Riesenberg D, Guthke R (1999) High-cell-density cultivation of microorganisms. Appl Microbiol Biotechnol 51:422–430

Rocha M, Mendes R, Rocha O, Rocha I, Ferreira EC (2014) Optimization of fed-batch fermentation processes with bio-inspired algorithms. Expert Syst Appl 41:2186–2195

Saharan K, Sarma MVRK, Prakash A, Johri BN, Bisaria VS, Sahai V (2011) Shelf-life enhancement of bio-inoculant formulation by optimizing the trace metals ions in the culture medium for production of DAPG using fluorescent pseudomonad R62. Enzym Microb Technol 48:33–38

Salehmin MNI, Annuar MSM, Chisti Y (2014) High cell density fed-batch fermentation for the production of a microbial lipase. Biochem Eng J 85:8–14

Sarkar D, Modak JM (2003) Optimisation of fed-batch bioreactors using genetic algorithms: two control variables. Comput Aided Chem Eng 14:1127–1132

Sarma MVRK, Gautam A, Kumar L, Saharan K, Kapoor A, Shrivastava N, Sahai V, Bisaria VS (2013) Bioprocess strategies for mass multiplication of and metabolite synthesis by plant growth promoting pseudomonads for agronomical applications. Process Biochem 48:1418–1424

Shukla PK, Pushpavanam S (1998) Optimisation of biochemical reactors—an analysis of different approximations of fed-batch operation. Chem Eng Sci 53:341–352

Sun Z, Ramsay JA, Guay M, Ramsay BA (2006) Automated feeding strategies for high-cell-density fed-batch cultivation of *Pseudomonas putida* KT2440. Appl Microbiol Biotechnol 71:423–431

Suzuki T, Mushiga Y, Yamane T, Shimizu S (1988) Mass production of lipase by fed-batch culture of *Pseudomonas fluorescens*. Appl Microbiol Biotechnol 27:417–422

Suzuki T, Yamane T, Shimizu S (1990) Phenomenological background and some preliminary trials of automated substrate supply in pH-stat modal fed-batch culture using a setpoint of high limit. J Ferment Bioeng 69:292–297

Travis J, Kring J (2006) LabVIEW for everyone: graphical programming made easy and fun, 3rd edn. Part of the National Instruments Virtual Instrumentation Series, Prentice Hall

Yamane T, Shimizu S (1984) Fed-batch techniques in microbial processes. In: Fiechter A (ed) Advances in biochemical engineering biotechnology, vol 30. Springer, Berlin, pp 147–194

Yee L, Blanch HW (1992) Recombinant protein expression in high cell density fed-batch cultures of *Escherichia coli*. Nat Biotechnol 10:1550–1556

Chapter 25
Protocol for Biocontrol of Soybean Cyst Nematode with Root Endophytic Fungi

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Abstract Plant-parasitic nematodes (PPNs) are major plant pests worldwide and cause severe economic and yield losses. The sedentary endoparasites, root-knot nematodes (RKNs, *Meloidogyne* spp.) and cyst nematodes (CNs, *Globodera* spp. and *Heterodera* spp.), are among the most economically damaging PPNs. *Piriformospora indica*, a plant growth-promoting fungus isolated from the Thar Deserts of western India, has been shown to protect a wide range of plants from various biotic and abiotic stresses. To evaluate the potential of *P. indica* to protect soybean (*Glycine max*) seedlings from damage by the soybean cyst nematode (SCN), we amended soil with two different concentrations of *P. indica* (2.5 % w/w, T1, and 5 % w/w, T2) and inoculated with second-stage juveniles (J_2s) of SCN in each treatment. After 60 days, abundance of nematode eggs was measured by calculating SCN egg population densities. We found that egg density/100 cubic cm soil was significantly decreased by 29.7 % and 36.7 %, respectively, in the soil amended with T1 and T2 compared to a control. Amendment with *P. indica* also had a strong growth- and yield-promoting effect in Soybean. Although root biomass was significantly decreased by 27.9 % and 33.5 % in the two treatments compared to the control, shoot biomass (dry weight) increased by 30.8 % and 8.2 % in the T1 and T2 treatments compared to the control. Additionally, plant development was accelerated and a 75 % increase in flowering was observed between the T1 treatment and the control. We conclude that *P. indica* used as a soil amendment decreases abundance of the SCN in soil and has plant growth-promoting properties that may help counteract yield losses due to plant-parasitic nematodes.

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25.1 Introduction

Plant-parasitic nematodes (PPNs) are small roundworms comprising about 4000 species infesting roots of thousands of plant species and causing tremendous crop yield losses worldwide (Blok et al. 2008). Because nematodes are difficult or impossible to see in the field, and their symptoms are often nonspecific, the damage they inflict is often attributed to other, more visible causes. Thus, farmers and researchers often underestimate their effects. A general assessment is that plant-parasitic nematodes reduce agricultural production by approximately 12–14 % worldwide (Postnikova et al. 2015), reducing production by millions of tonnes every year. This causes global agricultural losses amounting to an estimated US $173 billion annually (Elling 2013).

The amount of damage nematodes depends on a wide range of factors, such as their population density, the virulence of the species or strain, and the resistance (ability of the plant to resist infection by the nematode) or tolerance (ability of the plant to produce high yield despite nematode attack) of the host plant. Other factors also contribute to a lesser extent, including climate, water availability, soil conditions, soil fertility, and the presence of other pests and diseases (Coyne et al. 2007).

25.2 Sedentary Endoparasites

Sedentary endoparasitic nematodes are the nematodes that, once they have reached a feeding site inside the plant, cease to be mobile and feed from a fixed location. The sedentary endoparasites, root-knot nematodes (RKNs, *Meloidogyne* spp.) and cyst nematodes (CNs, *Globodera* spp. and *Heterodera* spp.), are among the most economically damaging PPNs. These parasites are obligate biotrophs that can feed only on the cytoplasm of living cells. Thus, both RKNs and CNs establish an intimate relationship with their host plants, inducing the redifferentiation of root cells into specialized multinucleate feeding cells (Quentin et al. 2013). They modify host root tissue to create a nutrient sink from which they feed (Eves-van den Akker et al. 2014).

25.3 Root-Knot Nematodes

Root-knot nematodes are obligate plant parasites that are distributed worldwide (Niu et al. 2016). These specialized sedentary endoparasites attack more than 3000 plant species and cause up to 5 % losses in global crop yield (Trudgill and Blok 2001). Their vernacular name comes from the galls (root knots) induced by *Meloidogyne* on the roots of their host plant. Mature females lay eggs in a protective gelatinous matrix which forms an egg mass. Egg masses are found on the root

surface or may be embedded in galls or plant tissue (e.g., potato tubers) and can contain up to 1000 eggs. After embryogenesis, the first-stage juvenile (J_1) molts within the egg to form the infective second-stage juvenile (J_2), which hatches from the egg. Many *Meloidogyne* species have a broad host range, and, in general, hatching is dependent solely on suitable temperature and moisture conditions, with no stimulus from host plants being required. J_2s usually penetrate the roots directly behind the root cap but can penetrate at any site. To facilitate penetration, J_2s use a combination of physical damage through thrusting of the stylet and breakdown of the cell wall by cellulolytic and pectinolytic enzymes.

25.4 Cyst Nematodes (*Heterodera* and *Globodera* spp.)

The cyst nematodes are obligate biotrophs and are of great economic importance throughout the world. The most damaging species include soybean cyst nematodes (SCNs) (*Heterodera glycines*), potato cyst nematodes (PCNs) (*Globodera pallida* and *G. rostochiensis*), and cereal cyst nematodes (CCNs) (including *Heterodera avenae* and *H. filipjevi*). Losses caused by CCNs are heavily dependent on environmental conditions, but can be in excess of 90 % in some environments (Nicol et al. 2011). PCNs have been estimated to cause losses of 9 % of total potato production worldwide (Turner and Rowe 2006). This nematode originated in South America, but has subsequently spread to nearly all major potato-growing regions of the world, and is still a major quarantine pathogen (Hockland et al. 2012).

The infective stage juveniles (J_2) of *H. schachtii* and other cyst nematodes hatch from eggs that are stored in the cyst, the modified dead body of the females (Hütten et al. 2015). This is the dormant stage of the life cycle, and in host-specific species, hatching occurs in response to host-derived chemical cues present in root diffusates (Perry 2002). The J_2 then locates its host, invades, and migrates destructively and intracellularly through the root until it reaches the inner cortex. Adult males leave the root to mate with females. The females continue to feed and when egg development is completed, they die and their body melanizes to form a cyst, enclosing hundreds of eggs, which constitute a survival stage that can remain viable for years in soils (Montarry et al. 2015; Bohlmann and Sobczak 2014). Particular problems are caused by the ability of cyst nematodes to survive for prolonged periods in the soil in the absence of a host (up to 20 years—Grainger 1964), making control by rotations difficult and eradication, once established, almost impossible.

25.5 Biocontrol of Nematodes

Although agronomic management practices are often deployed to manage plant parasitic nematodes, control with chemical nematicides is also used for high-value crops. Because chemical nematicides are toxic and persistent, they are a human

health risk and most are being phased out (Kumar et al. 2014). There is a great need for less toxic and environmentally sustainable solutions to nematode pests. Nematophagous fungi (including trapping, endoparasitic, and egg-parasitic fungi) are important natural enemies of nematode pests (Wang et al. 2009). While some nematophagous fungi directly parasitize nematodes, others may function through inducing plant defense responses or through production of secondary metabolites toxic to nematodes. Toxin-producing nematophagous fungi secrete toxins that immobilize nematodes before penetration of hyphae through the nematode cuticle (Lòpez-Llorca et al. 2008). Most of these fungi are Basidiomycota (e.g., *Pleurotus, Coprinus*) (Yang and Zhang 2014). *P. indica* also released some nematicidal compounds. Active compounds from culture filtrates of different endophytic fungi have also been described to have nematicidal or nematistatic potentials (Mani and Sethi 1984a, b; Cayrol et al. 1989; Cayrol and Djian 1990; Hallmann and Sikora 1996; Anke and Sterner 1997; Chen et al. 2000; Meyer et al. 2000; Daneshkhah et al. 2013).

25.6 Role of *P. indica* in Biological Control of Nematodes

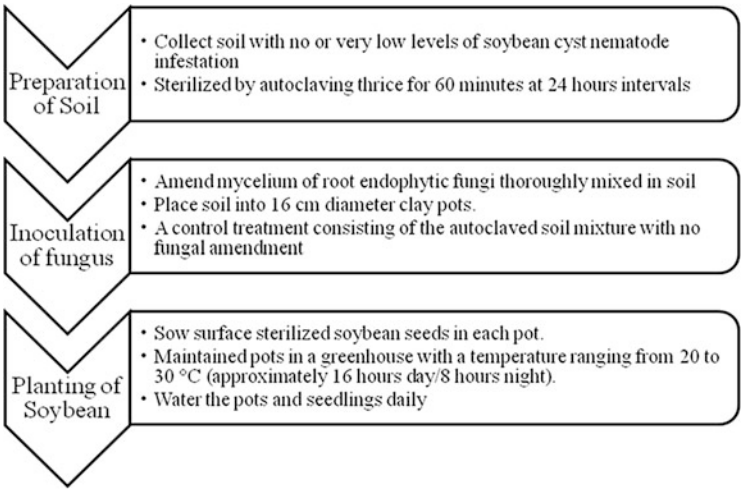
Piriformospora indica, a plant growth-promoting endophytic fungus isolated from the Thar Deserts of western India, has been shown to protect a wide range of plants from various biotic and abiotic stresses (Bajaj et al. 2014). Analysis for taxonomic position by molecular methods based on 18S rRNA sequences and by electron microscopy suggests that this fungus is related to the Hymenomycetes of the Basidiomycota (Varma et al. 1999). Recent evidence suggests that *P. indica* may also provide protection from belowground invertebrate pests such as nematodes. Daneshkhah et al. (2013) reported that plants precolonized with the biotrophic colonization stage of *P. indica* prior to inoculation with *Heterodera schachtii* led to a significant reduction in the number of nematode infection sites and disturbed nematode development. Potential mechanical barriers formed by the hyphae that may hinder J_2 migration or syncytium expansion play a secondary and rather minor role. Instead, they observed that in addition to direct colonization by the endophyte, treatment of the roots with a cell wall extract of the mycelia also inhibited nematode infection and development (Daneshkhah et al. 2013). This nematode-antagonistic activity could also be explained by production of secondary fungal metabolites and enzymes such as chitinases that feature toxicity against plant-parasitic nematodes (Shinya et al. 2008). Filtrate from *P. indica* cultures has been shown to contain compounds inhibitory to nematodes (Bajaj 2015).

25.7 Case Study

The soybean cyst nematode (*Heterodera glycines*) is a plant-parasitic nematode which is a major plant pest worldwide and causes severe economic and yield losses. *P. indica*, a plant growth-promoting fungus isolated from the Thar Deserts of western India, has been shown to protect a wide range of plants from various biotic and abiotic stresses. To evaluate the potential of *P. indica* to protect soybean seedlings from damage by the SCN, soil was amended with two different concentrations of *P. indica* (2.5 % as T1 and 5 % as T2 w/w) and inoculated with second-stage juveniles (J_2s) of SCN in each treatment. After 60 days, abundance of nematode eggs was measured by calculating SCN egg population densities (Bajaj et al. 2015) (Fig. 25.1).

Techniques to Study Biocontrol of SCN in Pot Trials

Preparation of Soil:



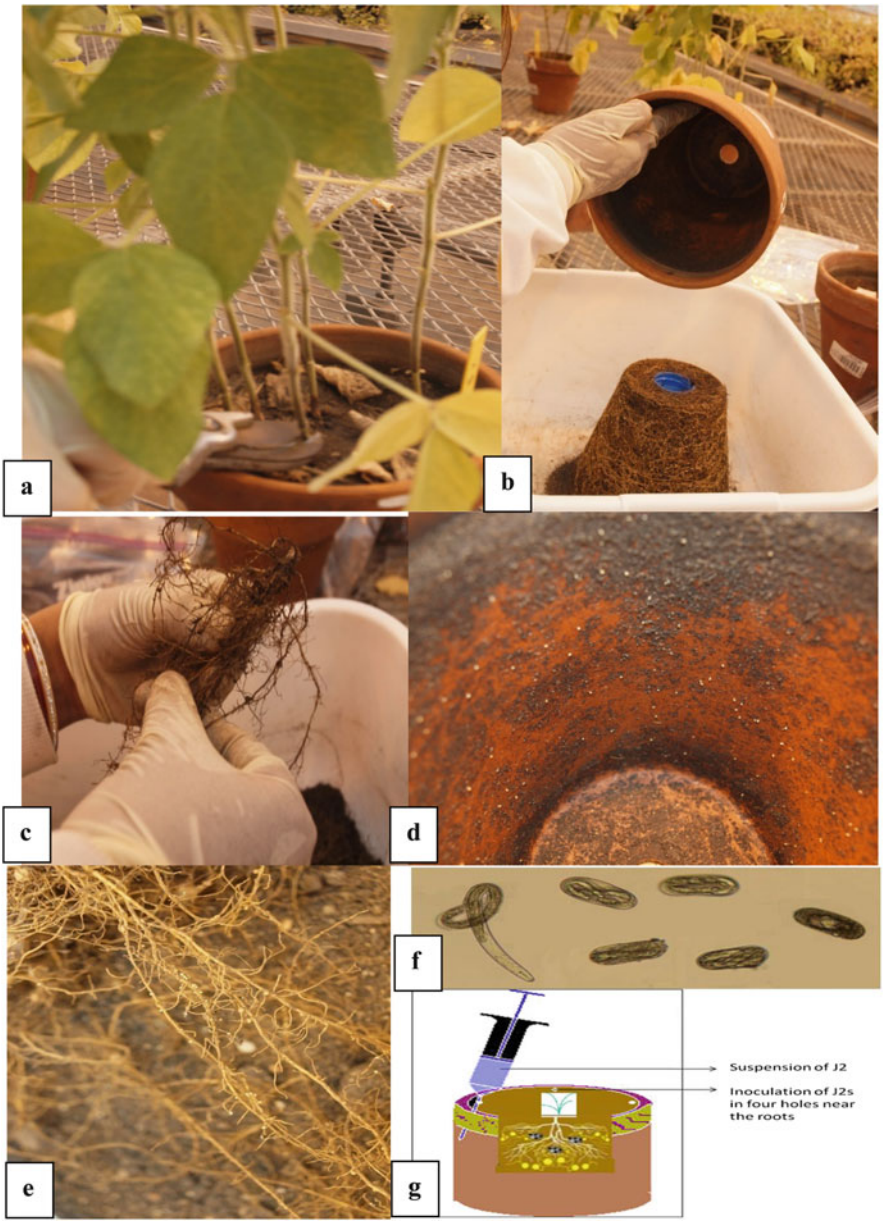
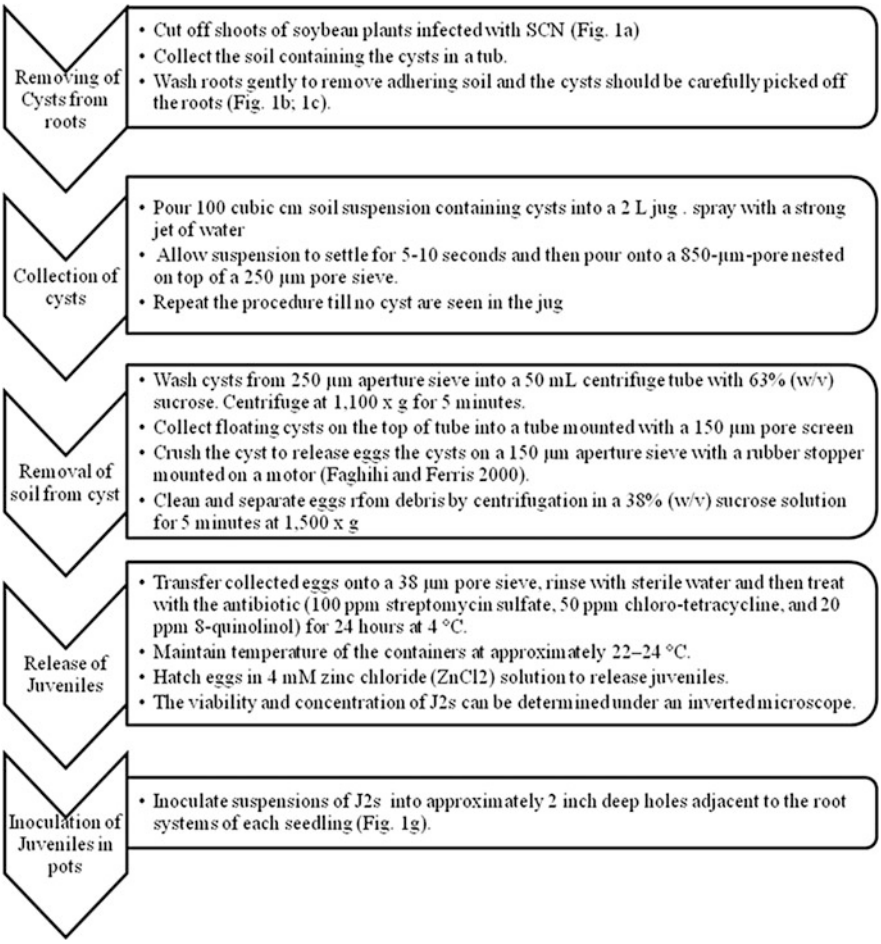


Fig. 25.1 Isolation of J_2 nematodes and inoculation into the pots. (**a**) Shoots were cut in dry soil; (**b**) the soil was collected into the tub; (**c**) the roots were properly cleared of adhered cysts; (**d**) the cysts attached to the pots; (**e**) the cysts attached to the roots; (**f**) the eggs of SCN seen under microscope; (**g**) inoculation of J_2s into the pot

Preparation and Inoculation of J_2s:



25.8 Fungal Root Colonization Percent

Roots should be washed in running tap water, cleaned by soaking in 10 % KOH for 4 days, acidified with 1 N HCl for 5 min, and then stained with lactophenol cotton blue. The roots can be observed under 630× optical microscope (Olympus BH2, Leeds Precision Instrument, Inc.). Estimation of percent colonization can be done using the gridline intersect method (Norris et al. 1994; Varma and Kharkwal 2009). Spores can be counted under a stereo binocular microscope.

Percentage root colonization was calculated with the formula: percent colonization = number of colonized roots × 100/number of roots observed.

25.9 Measurement of Egg Density

Plants should be harvested 60 days after inoculation of juveniles. SCN complete their life cycle in 60 days. The soil should be thoroughly mixed, and a subsample of 100 cubic cm (cc) soil (weight of approximately 190 g of soil ≈ 100 cc soil) should be collected in a 1 L beaker and soaked in 500 mL water for 30 min. The eggs should be isolated and cleaned using the procedure described above in the section "Preparation and inoculation of J_2s." The collected eggs should be diluted and the concentration of nematode eggs should be counted in a known sample volume using an inverted microscope in a petri plate with a grid. The total number of eggs can be calculated with the following formula:

$$\text{Total number of eggs} = \frac{\text{number of eggs counted in sample volume} \times \text{total volume of egg suspension}}{\text{sample volume of egg suspension counted}}$$

25.10 Colonization of Soybean Roots by *P. indica*

In this study, *P. indica* was inoculated onto the oilseed crop soybean in a controlled greenhouse trial in order to analyze its possible effects on growth, development, and pest resistance toward the SCN. Staining of soybean roots with lactophenol cotton blue revealed extensive root colonization by *P. indica* and formation of intracellular chlamydospores. However, colonization was confined to the root cortex. Chlamydospores were found as isolated spores, pairs, tetrads, long chains, and sometimes in clusters. Levels of root colonization ranged from 45 to 50 % at 60 days after planting.

25.11 Effects of *P. indica* on Growth and Development of Soybean

Soybean showed a positive interaction with *P. indica*, as demonstrated by increased shoot biomass and length of inoculated plants as compared to control plants (Table 25.1). Shoot length increased by 17.4 % in the T1 treatment compared to the control (Table 25.1). Similarly, the average shoot dry weight increased by

Table 25.1 Indicators of plant growth and nutrient status

Growth parameter	Control	T1	T2
% Root colonization± s.d.	0	46.04	48
	n/a	±0.03	±0.02
Shoot length (cm)± s.d.	38.16	44.80	38.86
	±3.40	±2.78*	±1.39
Chlorophyll (SPAD)± s.d.	28.78	29.38	28.80
	±0.42	±0.63	±0.54
Root fresh weight (g)± s.d.	37.03	26.78	25.87
	±4.03	±1.03*	±2.24**
Root dry weight (g)± s.d.	11.97	8.63	7.96
	±1.52	±0.69*	±1.01**
Shoot fresh weight (g)± s.d.	43.91	55.13	47.50
	±4.14	±8.01**	±2.71*
Shoot dry weight (g)± s.d.	13.73	17.97	16.11
	±1.00	±0.99**	±0.95*

Statistically significant differences in comparisons between each treatment and the control are indicated by * at $p = 0.05$ or ** at $p = 0.01$

Table 25.2 Indicators of plant development

Development parameter	Control	T1	T2
No. of branches ± s.d.	11.8 ± 1.5	13.5 ± 1.2	13 ± 0.8
No. of leaves ± s.d.	35 ± 4.5	41 ± 5.5	40 ± 4.6
No. of flowers ± s.d.	22.5 ± 2.6	39.5 ± 2.5**	28 ± 1.4*

Statistically significant differences in t-tests between each treatment and the control are indicated by * at $p = 0.05$ and ** at $p = 0.01$

30.8 % in the T1 treatment compared to control and by 8.2 % in the T2 treatment (Table 25.1). In contrast, the overall weight of colonized roots was lower than the weight of the uncolonized control roots. The root dry weights in the T1 and T2 treatments decreased by 27.9 % and 33.5 %, respectively, as compared to the control (Table 25.1). There was no detectable effect of the fungus on the chlorophyll content (Table 25.1).

Inoculation with *P. indica* also affected the timing and extent of plant development. The average number of leaves and branches increased by 17.2 % and 14.9 %, respectively, in the T1 as compared to control, although these differences were not statistically significant (Table 25.2). We also observed that *P. indica* not only induced development of the vegetative aerial part of the plant, but was also responsible for early maturation with respect to flowering. There was an increase of more than 75 % in flowers observed in the T1 compared to the control (Table 25.2). Similar results were observed for the T2 treatment with an increase

Table 25.3 Effects of *P. indica* on SCN egg density

Egg density	Control	T1	T2
Eggs/100 cc soil ± s.d.	1.548×10^6 $\pm 0.105 \times 10^6$	1.088×10^6 $\pm 0.062 \times 10^{6*}$	0.980×10^6 $\pm 0.054 \times 10^{6**}$
Eggs/g root wet weight ± s.d.	0.342×10^6 $\pm 0.045 \times 10^6$	0.330×10^6 $\pm 0.028 \times 10^6$	0.308×10^6 $\pm 0.019 \times 10^6$
Eggs/g dry root weight ± s.d.	0.106×10^6 $\pm 0.190 \times 10^6$	0.102×10^6 $\pm 0.066 \times 10^6$	0.100×10^6 $\pm 0.080 \times 10^6$

Statistically significant differences in t-tests between each treatment and the control are indicated by * at $p = 0.05$ and ** at $p = 0.01$

of 13.5 %, 10.6 %, and 24 % in a number of leaves, branches, and flowers, respectively (Table 25.2).

25.12 Effect of *P. indica* on SCN Egg Density

The number of SCN eggs per cubic cm (cc) soil, a common screening measure of SCN severity in agricultural fields, was significantly lower in the *P. indica*-amended pots (Table 25.3). There was a decrease of 29.7 % in the T1 treatment and 36.7 % in the T2 treatment (Table 25.3). Egg density per cc soil was also significantly reduced by 11.0 % between the T1 and the T2 treatments (Table 25.3). Egg density calculated as number of eggs/cc soil/gram root wet or dry weight also showed a trend of decreasing egg density with increasing *P. indica* in soil, although these comparisons were not statistically significant. For egg density/cc soil/gram root wet weight, the egg density decreased by 3.5 % from control to T1 and by 7.1 % from 2.5 % to T2. For egg density/cc soil/gram root dry weight, these decreases were 3.9 % and 1.9 %, respectively (Table 25.3).

25.13 Conclusion

Of the microorganisms that parasitize or prey on nematodes, fungi hold an important position and some of them have shown great potential as biocontrol agents (Jatala 1986; Stirling 1991). Amendment of soil with *P. indica* appeared to reduce egg production by the SCN while also stimulating growth and development of soybean plants. While further research is needed to address the specific mechanisms by which *P. indica* is able to lower levels of SCN eggs in soil while also enabling soybean plants to sustain higher yields in the presence of the SCN, it appeared that the fungus has potential applications for controlling levels of SCN infestation in agricultural soils.

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References

Anke H, Sterner O (1997) Nematicidal metabolites from higher fungi. Curr Org Chem 1:427–440

Bajaj R (2015) Effects of microbial sybiont on growth and pathogenic defense of selected plants. Ph.D. Thesis, Amity University Uttar Pradesh

Bajaj R, Agarwal A, Rajpal K, Asthana S, Kumar R, Prasad R, Kharkwal AC, Sherameti I, Oelmüller R, Varma A (2014) Co-cultivation of *Curcuma longa* with *Piriformospora indica* enhances the yield and active ingredients. Am J Curr Microbiol 2:6–17

Bajaj R, Hu W, Huang Y, Chen S, Prasad R, Varma A, Bushley KE (2015) The beneficial root endophyte *Piriformospora indica* reduces egg density of the soybean cyst nematode. Biol Control 90:193–199

Blok VC, Jones JT, Phillips MS, Trudgill DL (2008) Parasitism genes and host range disparities in biotrophic nematodes: the conundrum of polyphagy versus specialisation. Bioessays 30:249–259

Bohlmann H, Sobczak M (2014) The plant cell wall in the feeding sites of cyst nematodes. Front Plant Sci 5:89. doi:10.3389/fpls.2014.00089

Cayrol JC, Djian C (1990) Study of the toxicity of Fusarium roseum var. arthrosporioides to the nematode *Meloidogyne arenaria*. Comptes-rendus Acad Agric France 76:121–129

Cayrol JC, Djian C, Pijarowski L (1989) Study of the nematocidal properties of the culture filtrate of the nematophagous fungus *Paecilomyces liliacinus*. Revue Nèmatol 12:331–336

Chen SY, Dickson DW, Mitchell DJ (2000) Viability of *Heterodera glycines* exposed to fungal filtrates. J Nematol 32:190–197

Coyne DL, Nicol JM, Claudius-Cole B (2007) Practical plant nematology: a field and laboratory guide. IITA, Cotonou

Daneshkhah R, Cabello S, Rozanska E, Sobczak M, Grundler FMW, Wieczorek K, Hofmann J (2013) *Piriformospora indica* antagonizes cyst nematode infection and development in *Arabidopsis* roots. J Exp Bot 64:3763–3774

Elling AA (2013) Major emerging problems with minor *Meloidogyne* species. Phytopathology 103:1092–1102

Eves-van den Akker S, Lilley CJ, Danchin EG, Rancurel C, Cock PJ, Urwin PE, Jones JT (2014) The transcriptome of *Nacobbus aberrans* reveals insights into the evolution of sedentary endoparasitism in plant-parasitic nematodes. Genome Biol Evol 6:2181–2194

Faghihi J, Ferris JM (2000) An efficient new device to release eggs from *Heterodera glycines*. J Nematol 2:411–413

Grainger J (1964) Factors affecting the control of eelworm diseases. Nematologica 10:5–20

Hallmann J, Sikora R (1996) Toxicity of fungal endophyte secondary metabolites to plant parasitic nematodes and soil-borne plant pathogenic fungi. Eur J Plant Pathol 102:155–162

Hockland S, Niere B, Grenier E, Blok VC, Phillips MS, Den Nijs L, Anthoine G, Pickup J, Viaene N (2012) An evaluation of the implications of virulence in non-European populations of *Globodera pallida* and *G. rostochiensis* for potato cultivation in Europe. Nematology 14:1–13

Hütten M, Geukes M, Misas-Villamil JC, van der Hoorn RA, Grundler FM, Siddique S (2015) Activity profiling reveals changes in the diversity and activity of proteins in *Arabidopsis* roots in response to nematode infection. Plant Physiol Biochem 97:36–43

Jatala P (1986) Biological control of plant-parasitic nematodes. Annu Rev Phytopathol 24:453–489

Kumar M, Gantasala NP, Roychowdhury T, Thakur PK, Banakar P, Shukla RN, Jones MG, Rao U (2014) De novo transcriptome sequencing and analysis of the cereal cyst nematode, *Heterodera avenae*. PLoS One 9:e96311

Lòpez-Llorca LV, Macia-Vicente JG, Jansson HB (2008) Mode of action and interactions of nematophagous fungi. In: Ciancio A, Mukerji KG (eds) Integrated management and biocontrol of vegetable and grain crops nematodes. Springer, Dordrecht, pp 51–76

Mani A, Sethi CL (1984a) Effect of culture filtrates of *Fusarium oxysporum* f. sp. cicero and *Fusarium solani* on hatching and juvenile mobility of *Meloidogyne incognita*. Nematropica 14:139–144

Mani A, Sethi CL (1984b) Some characteristics of culture filtrate of *Fusarium solani* toxic to *Meloidogyne incognita*. Nematropica 14:121–129

Meyer SLF, Massoud SI, Chitwood DJ, Roberts DP (2000) Evaluation of *Trichoderma virens* and *Burkholderia cepacia* for antagonistic activity against root-knot nematode, *Meloidogyne incognita*. Nematology 2:871–879

Montarry J, Jan PL, Gracianne C, Overall AD, Bardou-Valette S, Olivier E, Fournet S, Grenier E, Petit EJ (2015) Heterozygote deficits in cyst plant-parasitic nematodes: possible causes and consequences. Mol Ecol 24:1654–1677

Nicol JM, Turner SJ, Coyne DL, den Nijs L, Hockland S, Maafi ZT (2011) Current nematode threats to world agriculture. In: Jones JT, Gheysen G, Fenoll C (eds) Genomics and molecular genetics of plant–nematode interactions. Springer, Heidelberg, pp 21–44

Niu J, Liu P, Liu Q, Chen C, Guo Q, Yin J, Yang G, Jian H (2016) Msp40 effector of root-knot nematode manipulates plant immunity to facilitate parasitism. Sci Rep 6:19443. doi:10.1038/srep19443

Norris JR, Read D, Varma AK (1994) Techniques for mycorrhizal research. Academic, London

Perry RN (2002) Hatching. In: Lee DL (ed) The biology of nematodes. Taylor and Francis, London, pp 147–169

Postnikova OA, Hult M, Shao J, Skantar A, Nemchinov LG (2015) Transcriptome analysis of resistant and susceptible alfalfa cultivars infected with root-knot nematode *Meloidogyne incognita*. PLoS One 10:e0118269

Quentin M, Abad P, Favery B (2013) Plant parasitic nematode effectors target host defense and nuclear functions to establish feeding cells. Front Plant Sci 4:53. doi:10.3389/fpls.2013.00053

Shinya R, Aiuchi D, Kushida A, Tani M, Kuramochi K, Koike M (2008) Effects of fungal culture filtrates of *Verticillium lecanii* (Lecanicillium spp) hybrid strains on *Heterodera glycines* eggs and juveniles. J Invertebr Pathol 97:291–297

Stirling GR (1991) Biological control of plant parasitic nematodes. Progress problems and prospects. CAB International, Wallingford, p. 282

Trudgill DL, Blok VC (2001) Apomictic, polyphagous root-knot nematodes: exceptionally successful and damaging biotrophic root pathogens. Annu Rev Phytopathol 39:53–77

Turner SJ, Rowe JA (2006) Cyst nematodes. In: Perry RN, Moens M (eds) Plant nematology. CAB International, Wallingford, pp 90–122

Varma A, Kharkwal AC (2009) Symbiotic fungi. Springer, Berlin

Varma A, Verma S, Sahay N, Bütehorn B, Franken P (1999) *Piriformospora indica* a cultivable plant-growth-promoting root endophyte. Appl Environ Microbiol 65:2741–2744

Wang B, Liu X, Wu W, Liu X, Li S (2009) Purification, characterization, and gene cloning of an alkaline serine protease from a highly virulent strain of the nematode-endoparasitic fungus *Hirsutella rhossiliensis*. Microbiol Res 164:665–673

Yang J, Zhang KQ (2014) Biological control of plant-parasitic nematodes by nematophagous fungi. In: Zhang KQ, Hyde KD (eds) Nematode-trapping fungi. Springer, Dordrecht, pp 231–262

Chapter 26
Spectrophotometric Assays to Evaluate the Rhizospheric Microbes Mediated Drought Tolerance in Plants

Jaagriti Tyagi and Ramesh N. Pudake

Abstract Drought stress is the most threatening environmental effect which limits the growth and productivity of plants globally. It is considered to cause oxidative stress in plants which results in the accumulation of reactive oxygen species. Oxidative stress is defined as an imbalance between antioxidants and reactive oxygen species (ROS) in response to any environmental stress. The antioxidant defence machinery protects plants against oxidative stress damages. Plants possess very efficient enzymatic (superoxide dismutase, SOD; catalase, CAT; polyphenol oxidase; guaiacol peroxidase), non-enzymatic (ascorbic acid, ASH; glutathione, GSH; phenolic compounds), and osmolyte (proline and soluble sugars) antioxidant defence systems which work in concert to control the cascades of uncontrolled oxidation and protect plant cells from oxidative damage by scavenging of ROS. Various soil microorganisms such as arbuscular-mycorrhizal fungi or plant growth-promoting rhizobacteria (PGPR) are the obligate symbionts that can improve plant tolerance to drought stress by increasing both plant nutrition and antioxidant defence system against the oxidative stress produced by water scarcity. In a natural soil, rhizosphere competence needs to be considered for successful interactions between these microorganisms and plants to overcome the environmental stress problem. In this chapter, we centred the assay/protocols to evaluate this relationship of antioxidant machinery and rhizospheric microbes by various spectrophotometric biochemical assays.

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26.1 Introduction

An unfavourable environment for a living organism is termed as abiotic stress and the ability of living organism to acclimate to such harsh environmental conditions can be called as abiotic stress resistance or stress tolerance (Bohnert et al. 2006). Plant growth and productivity are adversely affected by water stress such as reduction in leaf size, stem extension and root proliferation, water use efficiency, and disturbing plant water relations. During cellular respiration, the accretion of molecular O_2 in environment allows aerobic organism to utilize O_2 as the terminal electron acceptor. O_2 gives rise to prolific reactive excited states, like reactive oxygen species (ROS) and their derivatives during the various environmental stresses (Scandalios 2005). Furthermore, it causes changes in a number of physiological and biochemical processes governing plant growth and productivity (Daie and Patrick 1988). Assimilation of CO_2 by leaves is reduced mainly by stomatal closure, membrane damage, and disturbance in the activity of various enzymes, especially those of involved in CO_2 fixation and adenosine triphosphate (ATP) (Farooq et al. 2009). Plant tolerance to abiotic stress largely depends on their tolerance against oxidative stress, which is acquired largely by strong antioxidant defence systems (Al-Ghamdi 2009; Kocsy et al. 1996). The antioxidant system scavenges ROS through non-enzymatic antioxidants like ascorbic acid (AsA) and glutathione (GSH), phenolic compounds (vitamin E, flavonoids, phenolic acids), alkaloids, non-protein amino acids, α-tocopherols, carotenoids, chlorophyll derivatives, and enzymatic antioxidants like superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), glutathione reductase (GR), monodehydroascorbatereductase (MDHAR), dehydroascorbate reductase (DHAR), glutathione peroxidase (GPX), and glutathione S-transferase (GST) (Pang and Wang 2008; Gill and Tuteja 2010; Hasanuzzaman et al. 2012).

The results of many ongoing and past studies have shown that several plant species acquire stress tolerance by microbial associations during their growth. These associations are very critical for their survival. The ability of different plants to adapt to stress conditions is often considered to be dependent on their association with certain microbes. This give rise to a question how the plant can tolerate stress when associated with appropriate microbial partners. The physiological and biochemical analysis could make the mechanism of stress tolerance clear and could lead to new ways towards a sustainable agriculture. Plant growth-promoting rhizobacteria (PGPR) and endo- and ectomycorrhiza are the class of microbes that are associated with plant roots. These rhizospheric microbes might be involved in modifying the physiological processes in associated plant that results in "induced tolerance" in plants for drought stress. Thus, there is a need of studying plant response towards abiotic stress, and investigating the mechanism of microbes assisted tolerance is critical for achieving the target of developing tolerant plants.

So the methodology of biochemical assays of some of the stress markers is discussed in this chapter.

26.2 The Plant Growth Conditions and Drought Exposure Needed for Evaluation for Plant Responses

Seeds of plant chosen for study need to be surface sterilized for some time depending on size and seed parameters of different species. The common agents used for surface sterilization are sodium hypochlorite, mercuric chloride ($HgCl_2$), and ethanol. Finally, the seeds should be washed with distilled water thrice to remove trace amounts of adhering sterilizing agents from the seed surface. Seeds can be germinated in petri plates containing double-layered autoclaved filter paper with distilled water at 27 ± 2 °C. Three- to seven-day-old seedlings with uniform length should be transferred to pots and grown in a poly-house under controlled conditions.

Soil should be sieved in order to prepare uniform mixture of soil. Soil mixture must be autoclaved three times on alternative days at 121 °C (250 °F)/15 psi (pounds per square inch) for 60 min. Autoclaved soil must be placed loosely in pots in order to make uniform and soft bed. Pot should be weighed accurately to measure the uniform field capacity; and now pots are ready for planting. Make 1 cm deep hole in pots with the help of borer/or inoculating tips, and transfer seeds/seedlings in these holes without damaging roots. Seeds need to be distributed evenly onto the surface of soil. The microbial inoculum can be added to soil by soil application or seed treatment. After transferring in pots, plants need to be irrigated with tap water/distilled water at regular interval for pre-decided period, or until the plant reaches required growth. The moisture stress treatment can be given by getting the soil water status to 100 % (no-stress) and required water stress. For example, for 60 % (mild-stress) and 40 % (severe stress) of field capacity (FC), pots with plants are allowed to dry to the required level (for details, see Fig. 26.1).

After each day, the pots with plants need to be weighed and the amount of water lost by evapotranspiration can be supplied by re-watering to bring the soil water status back to 100, 60, and 40 % FC.

$$\%\text{Soil moisture} = \text{Fresh weight of soil} - \text{Dry weight of soil}/\text{dry weight of soil} \times 100$$

After suitable duration of drought treatment, plants are harvested and stored in −20 °C (Biochemical analysis) and −80 °C (Molecular analysis).

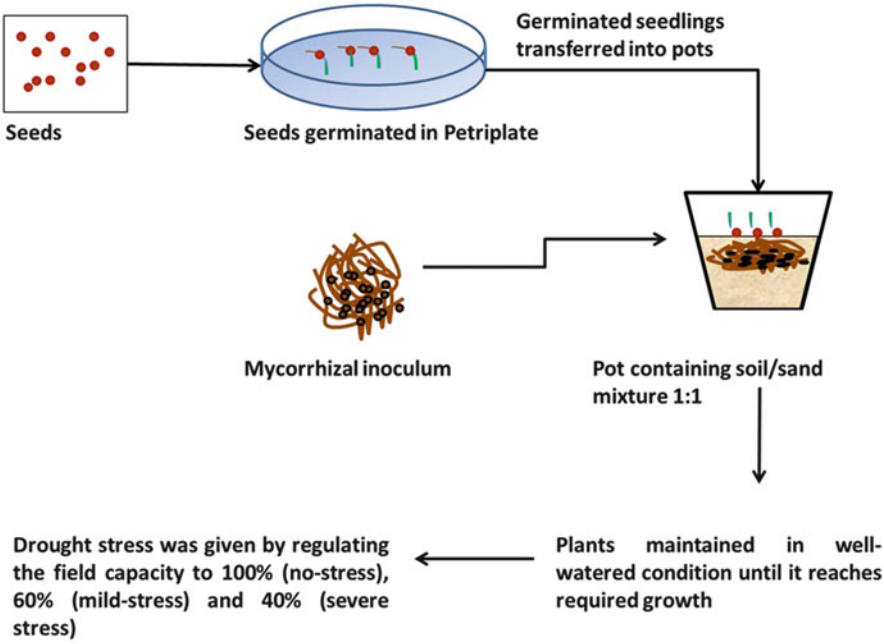

Fig. 26.1 Schematic diagram of procedure for seed germination and transfer to the pots containing soil–sand mixture and the microbial inoculum

26.3 Assessment of Drought Stress Response

For these experiments, materials like micropipette, centrifuge tubes, liquid nitrogen, motor and pestle, and distilled water are required. The equipment such as growth chamber, hot air oven, pH meter, UV–Vis spectrophotometer, and weighing balances are also required. Evaluation of drought stress in plant samples can be done by analysis of various biochemical parameters. The principles and methodology for each assay are discussed.

26.3.1 Estimation of Total Protein from Plant Samples

The Bradford reagent can be used to determine the concentration of proteins in solution (Bradford 1976). The procedure is based on the formation of a complex between the Brilliant Blue G dye and proteins in solution. The protein–dye complex shifts the absorption spectrum maximum of the dye from 465 to 595 nm. The amount of absorption is proportional to the protein present in sample.

The reagents needed for extraction of enzymes/protein from plants are deionized water, sodium phosphate buffer (50 mM, pH 7.5), phenylmethylsulfonyl fluoride

(PMSF, 1 mM), polyvinylpyrrolidone [8 % (w/v)], and Triton X-100 (0.01 %). For protein estimation, lyophilized 100 mg of plant sample is grounded in 0.01 M potassium phosphate buffer. Then mixture should be centrifuged immediately for 10 min at 12,000 rpm at 4 °C, and the supernatant is taken for protein estimation. Afterward, add 1 ml of Bradford reagent into 50 μl of extracted sample, and incubate the sample at room temperature for 5–10 min. Take the absorbance of sample at 595 nm against the blank processed in same manner. The protein dye complex is stable up to 60 min, so the absorbance of the samples must be recorded before 60 min time limit, and within 10 min of analysis of control samples. The bovine serum albumin (BSA) is used as standard for the construction of standard curve.

Calculate protein concentration using the equation given below:

$$X = \frac{\text{absorbance} + 0.6897}{1.5353}$$
$$= \text{Take antilog (Answer)}$$
$$= \mu g/10\ \mu l$$

(The values later can be converted into mg/mol).

26.3.2 Quantification of the Enzymatic Antioxidant Activity

In this chapter, we have also provided the details of enzyme activity that are measured spectrophotometrically to give researchers an easy and accurate way to understand an important part of defense against oxidative stress. All enzyme assays can be conducted using leaf or root tissues from the plants which are cocultivated with microbes under drought stress.

26.3.2.1 Estimation Superoxide Dismutase (SOD) Enzyme

SOD enzyme causes autoxidation of pyrogallol (1,2,3-benzenetriol) rapidly in alkaline solution, and the reaction has been employed for the removal of oxygen from gases. The autoxidation of pyrogallol was investigated in the presence of EDTA in pH range of 7.9–10.6. The rate of autoxidation increases with increasing pH. The enzyme superoxide dismutase almost inhibited 99 % of the reaction, indicating the total dependence on the participation of superoxide anion radical, $O_2^{\cdot -}$ in the reaction.

Reagents needed for the estimation of SOD enzyme: Tris-HCL buffer (0.1 M, pH 8.2), EDTA (10 mM), Pyrogallol (7.2 mM), and HCL (1 N) in deionized water.

Enzyme assay: The method used for monitoring the SOD activity is given by Marklund and Marklund (1974). Briefly, take 3 ml of reaction mixture containing Tris-HCL buffer and 10 mM EDTA (EDTA is difficult to solubilize in water, so the pH of the solution need to be increased more than eight using NaOH in order to

solubilize these salts). To this reaction mixture 100 µl of enzyme extract should be added rapidly, mixed well, and allowed to incubate at 25 °C for 10 min. The reaction is stopped by adding 50 µl of 10 mM HCL, and absorbance is measured at 420 nm. Calculate the percentage of SOD activity by the following formula:

$$\text{SOD activity } (\%) = \left[1 - (\text{A} - \text{B})/\text{C}\right] \times 100$$

The extracted sample added with (A) or without pyrogallol (B), and control (C) was represented by buffer solution added with pyrogallol.

26.3.2.2 Estimation of Catalase (CAT) Enzyme

Plant cells perform several metabolic processes by which hydrogen peroxide is produced as a harmful by-product. In most living organisms that are exposed to oxygen, the plant cells use catalase enzyme to speed up the process and breaking down the harmful hydrogen peroxide to the less dangerous oxygen gas and water molecules.

The continuous spectrophotometric rate reduction is based on the following equation:

$$2\text{H}_2\text{O}_2 \xrightarrow{\text{CAT}} 2\ \text{H}_2\text{O} + \ \text{O}_2$$

Reagents needed for the estimation of catalase enzyme: Potassium phosphate buffer 50 mM and hydrogen peroxide solution (30 %).

Enzyme Extraction: Catalase activity can be measured by the method suggested by Luck (1974). For this, lyophilize 0.5 g of tissue in a precooled mortar and pestle and add 1.5 ml of potassium phosphate buffer. Centrifuge this resulting suspension at 12,000 rpm for 20 min at 4 °C, and then use this extracted supernatant for enzyme assay.

Enzyme Assay: To estimate catalase enzyme, take 3 ml of H_2O_2-phosphate buffer in an experimental cuvette and 40 µl of enzyme extract is rapidly added in it. The enzyme solution containing H_2O_2-free phosphate buffer serve as control. The UV absorption of hydrogen peroxide is measured at 240 nm, whose absorbance decreases when degraded by the catalase enzyme. From the decrease in absorbance, the enzyme activity can be calculated as: one unit of catalase will decompose 1.0 µmole of H_2O_2 per minute at pH 7.0 at 25 °C, while the H_2O_2 concentration falls from 10.3 to 9.2 mM. The rate of disappearance of H_2O_2 is followed by observing the rate of decrease in the absorbance at 240 nm.

26.3.2.3 Estimation of Polyphenol Oxidase (PPO) Enzyme

Phenol oxidases are copper containing proteins that catalyze the aerobic oxidation of phenolic substrates to quinines, which are autoxidized to dark brown pigments known as melanins. These enzymes are released by the broken cells, and they catalyze the reaction between colorless molecules called polyphenols and molecular oxygen. These can be estimated spectrophotometrically at 495 nm. Catechol is one of the substrate for these enzymes, so it is alternatively called as "catechol oxidases". Catechol is first oxidized to the orange compound benzoquinone which is then converted to melanins. The conversion to melanin is spontaneous but slow.

$$\text{Catechol} + \text{Oxygen} \rightarrow \text{Benzoquinone} + \text{Water} \rightarrow \text{Melanins}$$

Reagents needed for the estimation of enzyme: Catechol (50 mM) and potassium phosphate buffer (100 mM, pH 6.0).

Enzyme assay: Method of Cavalcanti et al. (2007) is used for the estimation of PPO enzymes. The reaction mixture contains 100 mM potassium phosphate buffer (pH 6.8), 2.5 ml of 25 mM catechol, and 50 µl enzymatic extract. Measure the increase in absorbance of the reaction mixture at 410 nm by monitoring the oxidation of catechol. A value of 1300 M^{-1} cm^{-1} was employed for the molar extinction coefficient of *o*-Quinone (Gauillard et al. 1993). One unit of enzyme activity was expressed as µmol product formed/min/mg of protein.

26.3.2.4 Estimation of Guaiacol Peroxidase (GPOX) Enzyme

This method is based on monitoring GPOX scavenging activity by using guaiacol as a hydrogen donor. Presence of the enzyme is checked by mixing the extract with $H_2O + O_2$ and 2-methyoxyphenol which is commonly known as guaiacol. Peroxidase converts H_2O_2 to H_2O and O_2. If the enzyme is present, the peroxide is quickly converted to water and oxygen, and the oxygen reacts with the guaiacol to produce a brown product, oxidized guaiacol.

$$2H_2O_2 \xrightarrow{\text{GPOX}} H_2O + O_2$$

The reaction serves to convert toxic hydrogen peroxide (H_2O_2), a product of metabolism, into water and oxygen. The dye guaiacol (extracted from the guaiacum tree of Central and South America and the Caribbean) binds to peroxidase and becomes oxidized as the hydrogen peroxide is reduced to water. The complete reaction is as follows:

$$\underset{\text{Colourless}}{H_2O_2 + 4 - \text{guaiacol(reduced)}} \xrightarrow{\text{GPOX}} 8H_2O + \underset{\text{Brown}}{\text{Tetraguaiacol(oxidized)}}$$

Reagents needed for the estimation of enzyme: Guaiacol (40 mM), H_2O_2 (10 mM), and potassium phosphate buffer (100 mM, pH 6.5).

Enzyme assay: GPOX enzyme can be estimated according to the method suggested by Zhang et al. (2010). In 6 ml of reaction mixture, add 1 ml of 40 mM guaiacol (v/v), 1 ml of 10 mM H_2O_2 (v/v), and 4 ml of 100 mM potassium phosphate buffer (pH 6.5). Then 10 µl enzyme extract should be added rapidly to initiate the reaction. The blank sample must contain the same reaction mixture, without the enzyme extract. The absorbance should be recorded at 436 nm and the results are expressed in nM/min/mg of protein using an extinction coefficient of 25.5 mM^{-1} cm^{-1}.

Note: Guaiacol smells really bad. The whole process has to be conducted in fume hood.

26.3.3 Quantification of the Non-enzymatic Antioxidant Activity During Drought Stress

26.3.3.1 Estimation of Ascorbic acid (AsA) Content

Ascorbate (L-ascorbic acid) is a critical metabolite, which functions as a major redox buffer in plant cell. It is involved in various mechanisms such as regulation of cell division and growth (Horemans et al. 2000; Potters et al. 2002), regeneration of antioxidants, cofactor for enzymes including dioxygenases, ascorbate peroxidase, and violaxanthinde-epoxidase (Noctor et al. 2002), and protects cells and organelles from oxidative damage by scavenging reactive oxygen species and hydrogen peroxide. Ascorbate acts as antioxidant due to its involvement as donor of electrons in a wide range of enzymatic and non-enzymatic biochemical reactions (Noctor and Foyer 1998). Ascorbate is converted to dehydroascorbate by the treatment with 2,4 nitrophenyl hydrazine (DNPH) to form osazone, which dissolves in sulphuric acid to give an orange-coloured solution, and absorbance can be measured spectrophotometrically at 540 nm.

Reagents required for the estimation of AsA: 5 % (v/v) sulphosalicylic acid and 10 % thiourea.

Extraction Procedure: AsA content can be measured as described by Foyer et al. (1983). Lyophilize 0.1 g of fresh sample in a mortar containing 3 ml of 5 % (v/v) sulphosalicylic acid. Vortex it for few seconds, and centrifuge this resulting homogenate at 10,000 rpm for 15 min at 4° C. Add a pinch of activated charcoal in the resulting supernatants and mix thoroughly. After 5–10 min, centrifuge the mixture again to remove charcoal residue, and resulting supernatant is used for estimation of AsA content.

Assay: Add 0.5 ml of DNPH reagent to 0.5 ml of extracted supernatant, followed by 2 drops of 10 % thiourea solution. Vortex the mixture for few seconds and incubate the sample at 37 °C for 3 h. Dissolve the osazones formed in 2.5 ml of 85 % sulphuric acid, under cool conditions. For blank sample, sulphuric acid is added before the addition of DNPH and thiourea. Read the absorbance at 540 nm. Calculate the ascorbate concentration as mg ascorbate/g of leaf.

Note: Concentrated H_2SO_4 is corrosive and toxic when inhaled and when in contact with the skin. Protect skin and wear gloves and a lab coat; use in a fume hood.

26.3.3.2 Estimation of Chlorophyll Content

As chlorophyll is the major component of chloroplast required for photosynthesis; its content is reduced in plants due to limited supply of water (Paknejad et al. 2006). This reduction is a typical symptom of oxidative stress which results in the photo-oxidation and chlorophyll degradation (Anjum et al. 2011). Drought stress affects the photosynthesis process due to low availability of CO_2 which causes low stomatal and mesophyll conductance (Flexas et al. 2008) and/or impairs carbon assimilation metabolism (Peeva and Cornic 2009). Stomatal closure and lower light use efficiency are an early response to drought and an efficient way to reduce water loss in water-limiting environments. Chloroplast are readily extracted in organic solvents such as acetone, ether, and dimethyl sulfoxide.

Reagents: DMSO (Dimethyl sulfoxide).

Procedure:

Estimate the chlorophyll concentration by following the method of Hiscox and Israelstam (1979). Weigh 0.1 g of leaf sample and cut into small pieces with the help of scissor. Then, add 7 ml of dimethyl sulfoxide (DMSO) in test tubes containing 0.1 g of green-leaf tissue. Keep the test tubes in water bath at 65 °C for 30 min (expand time until green tissue becomes white). Then cool the tubes at room temperature; filter the resulting leaf sample, and make up volume upto 10 ml by adding DMSO. After vortexing for a few seconds, measure the absorbance at 645 and 663 nm. Calibrate spectrophotometer at zero absorbance using a blank of pure DMSO. The amount of chlorophyll present in the extract is calculated in mg of chlorophyll per gram tissue according to the following equation (Arnon 1949).

$$\text{Chla}\left(\text{g l}^{-1}\right) = 0.0127 \times \text{A663} - 0.00269 \times \text{A645};$$

$$\text{Chlb}\left(\text{g l}^{-1}\right) = 0.0229 \times \text{A645} - 0.00468 \times \text{A663};$$

$$\text{Tot Chl}\ \left(\text{g l}^{-1}\right) = 0.0202 \times \text{A645} + 0.00802 \times \text{A663}.$$

Notes:

1. DMSO is a hazardous substance, before handling, ensure nitrile gloves, lab coat, and safety glasses are worn. All work with DMSO must done under a fume hood.
2. To avoid the decomposition of chlorophyll pigments, take OD after 20 min of extraction procedure completed.
3. Discard major veins and any tough, fibrous tissue.

4. Avoid the exposure of leaf materials to direct sun light (take care to avoid the excessive loss of moisture from leaf by preserving them in distilled water)

26.3.3.3 Malondialdehyde (MDA) Content Estimation (Lipid peroxidation)

Lipid peroxidation is a well-established mechanism of cellular injury in plants and animals. Membranes are very prone to attack by reactive oxygen species (ROS), so lipid peroxidation is used as an indicator of oxidative stress in cells and tissues (Hodges et al. 1999). Lipid peroxides are unstable and decompose to form a complex series of compounds including reactive carbonyl compounds, especially certain aldehydes (malondialdehyde, MDA), 4-hydroxy-2-nonenal (4-HNE) that damage cells by the binding of the free amino groups of amino acids of proteins. This type of peroxidation is a chemical process, in which unsaturated fatty acids of lipids are damaged by free radicals and oxygen under lipoperoxides formation. Malondialdehyde is a secondary end product of the oxidation of polyunsaturated fatty acids and reacts with thiobarbituric acid (TBA) to yield a pinkish-red chromagen with maximal absorbance at 532 nm (Hodges et al. 1999).

Reagents required for the estimation of MDA content: Thiobarbituric acid (0.37 %), trichloroacetic acid (20 %), malondialdehyde, and HCL (0.25 N).

Procedure: 0.1 g of lyophilized plant material is transferred into a glass test tube containing 1 ml 5 % (w/v) TCA and thoroughly mix the sample. Centrifuge homogenized samples at 10,000 rpm for 10 min. Add 4 ml of 20 % TCA containing 0.37 % TBA to the supernatant and mix well. Boil the mixture at 95 °C for 15 min and quickly cool on ice. TBA can interact with MDA and results in red compound in acidic buffer. MDA can be calculated by measuring the density of the resulting red compound with spectrophotometer at 532 nm. To generate a standard curve, a serial concentration of MDA is made from 1–50 μM.

26.3.3.4 Total Phenol Estimation

Phenols are the major group of plant's secondary metabolites; and are the major antioxidant molecules. Aromatic ring with –OH or $–OCH_3$ substituents present in polyphenols contributes to their biological activity, including antioxidant action. A plant system contains two types of phenolics: cell wall phenolics, -lignins that provide mechanical barrier against penetration of biotic stressors and mechanical strength to plants under abiotic stresses, and soluble phenolics, which are ROS scavengers. The soluble phenolics are also used by peroxidases to detoxify ROS because the electron reduction potential of the phenolic radical is lower than the electron reduction potential of oxygen radicals (Grace and Logan 2000) and oxygen radicals are more reactive than phenoxyl radicals (Bors et al. 1990). Polyphenols can act as chelators for transition metal ions, can directly scavenge molecular species of active oxygen, and can inhibit lipid peroxidation by trapping the lipid

alkoxyl radical. Estimation of phenols using Folin-Ciocalteu's reagent is based on the reaction between benzene ring of phenols and an oxidizing agent phosphomolybdate in Folin-ciocalteu reagent which results in the formation of a blue complex (Bray and Thorpe 1954).

Reagents required for the estimation of total phenol content: Methanol (80 %), Folin-Ciocalteu reagent (1 N), sodium carbonate (700 mM), and gallic acid monohydrate.

Extraction Procedure: Harvested plants need to be shade dried for 5–7 days. Extract 100 mg of dry plant material with 4 ml of 80 % methanol. Place the phenol extract on an orbital shaker and incubate at room temperature for 2 h before phenol analysis (extraction should be done in brown test tubes to prevent oxidation). Keep the tubes in water bath at 30 °C for 30 min. Centrifuge the extract at 10,000 rpm, and collect supernatant in new test tube. Extract the pellet again with 2 ml of 80 % methanol, and after centrifugation the supernatant is added to the earlier supernatant. Other compounds such as chlorophyll and lipids that can disturb the colour reaction can be extracted with chloroform and 2.5 ml deionized water. Remove the chloroform by centrifugation for 10 min at 5000 rpm. The methanol water fraction is ready to be used for determination total phenolic content. Remaining samples can be used for extraction for insoluble sugars (fructans and starch).

Assay protocol: Prepare blank by adding 500 µl of methanol, 2.5 ml 10 % Folin-Ciocalteu's reagent dissolved in water, and 2.5 ml of $NaHCO_3$. The same procedure was repeated for the standard solution of gallic acid, and the calibration line is constructed. The content of phenolics in extracts is expressed in terms of gallic acid equivalent (mg of GA/g of extract).

Notes and troubleshooting tips: All reagents and solvents should be of analytical grade or of pure quality. Extraction should be done in dark-coloured test tubes to prevent oxidation. The F–C reagent should be added before the alkali to avoid the air oxidation of phenol. Deviation in the blue colour of samples results due to interference of glucose and fructose. Protein interference can be removed by TCA (Trichloro acetic acid).

Hazards: Gallic acid can cause irritation to eyes, respiratory system, and skin, so always wear gloves, lab coat, and goggles, and work in fume hood as required.

26.3.3.5 Estimation of Flavonoid Content

Flavonoids are the major secondary metabolites of plant, which accumulate in response to environmental stress such as biotic and abiotic stress (Saito et al. 2013). These antioxidants help in the scavenging of H_2O_2 and H_2O_2 generated hydroxyl radicals which are located in the nucleus of mesophyll cells (Agati et al. 2012). Flavanoids have the capacity to scavenge free radical due to the reduction potentials, and the consequent radical forms are lower than those of alkyl peroxyl radicals and the superoxide radical.

Reagents needed for the estimation of flavonoid content: 10 % aluminium chloride solution, 1 M potassium acetate, and methanol (80 %).

Procedure: Ordonez et al. (2006) had described the methodology to estimate total flavonoid contents of plants. This method is based on the formation of a stable complex between aluminium chloride and keto and hydroxyl groups of flavones and flavonoids. Extraction procedure for flavonoid is same as described in total phenol estimation. For assay by spectroscopy, add 0.1 ml of methanolic extracts with 0.1 ml of 10 % aluminium chloride prepared in methanol. Shortly in this mixture, add 0.1 ml of 1 M potassium acetate and 2.8 ml of distilled water. Thoroughly mix the resultant mixture and incubate for 30 min at room temperature. Yellow colour developed after incubation indicates the presence of flavonoid. Measure the absorbance of sample at 414 nm using UV–VIS spectrophotometer. Total flavonoid content is calculated as quercetin equivalent (mg/g).

Hazards: Anhydrous $AlCl_3$ reacts vigorously with bases, so suitable precautions are required. It can cause irritation to the eyes, skin, and the respiratory system if inhaled or on contact.

26.3.3.6 Proline Content Estimation

Proline, an amino acid plays key roles in plants such as redox homeostasis, osmotic regulation, protection of cell against various biotic and abiotic stresses, and cell signalling mechanism. One of the potentially important mechanisms of drought tolerance is osmotic regulation, which can be achieved by the accumulation of osmolytes like proline. It allows cell enlargement and plant growth during severe drought stress and allows stomata to remain partially open and CO_2 assimilation to continue during drought stress (Hare et al. 1998). Proline helps the cells to maintain their dehydrated state and the structural integrity of the membranes so as to provide resistance against drought and cellular dehydration (Ramanjulu and Bartels 2002). Ninhydrin (2,2-dihydroxyindane-1,3-dione) is extensively used to assay primary and secondary amines. At neutral pH, it destroys each primary α-amino acid and also reacts with the released NH_3 to form a deep purple chromogen referred to as Ruhemann's Purple, which has a maximum absorption at about 570 nm.

Procedure: Prepare reagents like a solution of 3 % sulphosalicylic acid, proline, ninhydrin, glacial acetic acid triorthophosphate (6 M), and toluene required for the estimation of proline. Proceed proline colorimetric determination according to Bates et al. (1973) and Marin Velazquez et al. (2010).

Lyophilize 0.1 g of plant material in 2 ml of 3 % aqueous sulphosalicylic acid and centrifuge at 12,000 rpm for 10 min for extraction. Resulting supernatant reacts with 1 ml acid-ninhydrin and 1 ml of glacial acetic acid in a test tube. Vortex the reaction mixture thoroughly and keep in boiling water bath for 1 h at 100 °C. Then the reaction is terminated on an ice bath (5–10 min). After cooling, the reaction mixture is extracted with 2 ml toluene, mixed vigorously and left at room temperature for 30 min until separation of the two phases. The chromophore-containing toluene (1 ml, upper phase) is warmed to room temperature and measure the optical density of the sample at 520 nm using toluene as a blank. The proline concentration is determined from a standard curve using D-Proline.

Role of reagents:

Sulfosalicylic acid causes the precipitation of protein which causes larger protein molecules to aggregate; that can then be removed by centrifugation leaving only free amino acid in the extract.

Ninhydrin reaction depends upon the pH and spectrum. Proline forms different reaction product with ninhydrin, and acidity is required to maintain the ninhydrin in stable and soluble form.

Toluene is an important organic, water-insoluble solvent. Amino acid–ninhydrin product is soluble in toluene phase and can be extracted easily.

pH	Spectrum (nm)	Colour
Neutral pH	550	Red
Acidic pH	520	Red
Alkaline pH	412	Blue

Care and troubleshooting: The experiment is always done at room temperature. The colour intensity is stable only for 1 , and the relationship between the amino acid concentration and absorbance is linear in the range 0.02–0.1 mM per mL of proline. Acid-ninhydrin reagent can't be stored for more than 24 h. As ninhydrin irritates the skin and respiratory system, it requires adequate precautions during handling. Inhaling toluene has potential to cause severe neurological harm, so always wear mask before performing assay.

26.3.3.7 Reduced Glutathione Estimation

Reduced glutathione (GSH) is a multifunctional antioxidant molecule which protects the plants against environmental stress by maintaining the cell in redox state (Tausz et al. 2001; Han et al. 2013). It protects the integrity of the cellular plasma membrane by maintaining α-tocopherol and xeaxanthin in the reduced state as well as protects proteins from denaturation caused by the oxidation of protein thiol groups (Paradiso et al. 2008). Glutathione has the ability to direct scavenge ROS and act as substrate for glutathione peroxidase and glutathione-S-transferase enzyme. These enzymes also help in ROS quenching. Reduced glutathione (GSH) is measured by its reaction with DTNB (5,5′-dithiobis-2-nitrobenzoic acid) (Ellman's reaction) to give a yellow-coloured product at 412 nm.

Reagents required for the estimation of GSH: 100 mM phosphate buffer (pH 7.0), DTNB (0.6 mM in 100 mM phosphate buffer), TCA (5 %), and Standard GSH (10 nM/ml in 5 % TCA).

Extraction Procedure: The method used for monitoring the GSH activity is proposed by Moron et al. (1979). A homogenate is prepared with 0.1 g of the plant sample with 1 ml of 5 % TCA. The precipitated protein is centrifuged at 1000 rpm for 10 min.

Estimation of GSH: The supernatant (100ul) is used for the estimation of GSH. The supernatant (100 μl) is made up to 1.0 ml with 100 mM potassium phosphate

buffer (pH 7.0). Two ml of freshly prepared DTNB solution is added and vortexed thoroughly. The intensity of the yellow colour developed is measured in a spectrophotometer at 412 nm after 10 min. The values are expressed as nM GSH/g sample. Standard GSH corresponding to concentrations ranging between 2 and 10 nM are prepared.

Hazards: 5-5′-Dithiobis(2-nitrobenzoic acid) irritates the eyes, respiratory system, and skin. In case of contact with eyes, rinse immediately with copious amounts of water and seek medical advice. Wear suitable protective clothing.

26.3.3.8 Total Soluble Sugars (TSS) Estimation

Sugars are the main product of photosynthesis due to its intimate involvement in growth, development, storage, signalling mechanism, and stress acclimation. Accumulation of soluble sugars in plants has various functions such as maintaining the turgidity in leaf, preventing the dehydration of proteins and cell membranes, and increased protective enzymes under stress condition (Sawhney and Singh 2002). The breakdown of cell wall polysaccharides (e.g. oligogalacturonides) (Camejo et al. 2012) might therefore also generate sugar signals under stress (Bolouri-Moghaddam et al. 2010). Carbohydrate is first hydrolysed into simple sugars using dilute HCL. In hot acidic medium, glucose is dehydrated to hydroxymethyl furfural, resulting in the formation of green coloured product with anthrone reagent.

Reagents: Standards of D-Glucose, Thiourea, Anthrone reagent, Ethanol (96 %), Sulphuric (98 %) acid, and HCL (2.5 N).

Procedure: 0.1 g of lyophilized plant material extracted with 4 ml of 96 % ethanol. Keep the test tubes in boiling water bath for 30 min. After centrifugation for 15 min at 10,000 rpm, collect the extract, and add 4 ml of anthrone reagent to this 0.2 ml of the ethanolic extract. Vortex the mixture thoroughly and keep the test tubes in boiling water bath for 4–5 min. After cooling on ice (4–5 min), allow the test tubes to warm up at room temperature. Read the green to dark green colour (leaves) and brown (roots) at 630 nm. Prepare the glucose standard from 1 to 100 µg/ml. A standard graph is drawn by taking the concentration of glucose on the *X* axis and spectrophotometer reading on the Y axis. From the graph, the concentration of glucose in the sample is calculated.

Note and Troubleshooting Tips: Prepare a fresh anthrone reagent, and store in dark-coloured bottles to prevent oxidation. Soluble sugars extracted from freeze-dried plant material because in oven-dried material, caramelization occurs which leads to an underestimation of soluble sugars. Colour development varies with the different sugars (Green, dark green, blue, etc.).

26.4 Conclusion

The rhizospheric microbes possess a synergistic effect of promoting plant growth and improving biotic and abiotic stress resistance as well (Tanaka et al. 2006). Plant growth-promoting bacteria (PGPB), as component of soil microbiota, have the potential role of improving the establishment of plant species under arid soil conditions (Hayat et al. 2010). Also Mycorrhiza have numerous beneficial effects on plants, including enhanced nutrient uptake, plant growth, and resistance to abiotic stresses, such as salinity (Sheng et al. 2008) and drought stress (Wu and Xia 2006). There are some reports indicating that mycorrhizal inoculation restricts the excessive production of ROS by enhancing antioxidant activity enzymes, such as SOD, POD, and CAT; and lower malondialdehyde (MDA) concentration compared to non-inoculated control plants. (Wu et al. 2013). But the underlying mechanisms for rhizospheric microbes mediated plant resistance to drought conditions have not yet been clearly elucidated. The methodology explained in this chapter will be useful tool to understand the mechanism.

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References

Agati G, Azzarello E, Pollastri S, Tattini M (2012) Flavonoids as antioxidants in plants: location and functional significance. Plant Sci 196:67–76

Al-Ghamdi AA (2009) Evaluation of oxidative stress tolerance in two wheat (*Triticum aestivum*) cultivars in response to drought. Int J Agric Biol 11:7–12

Anjum SA, Xie XY, Wang LC, Saleem MF, Man C, Lei W (2011) Morphological, physiological and biochemical responses of plants to drought stress. Afr J Agric Res 6:2026–2032

Arnon DI (1949) Copper enzymes in isolated chloroplasts. Polyphenoloxidase in *Beta vulgaris*. Plant Physiol 24:1–15

Bates LS, Waldren RP, Teare ID (1973) Rapid determination of free proline for water-stress studies. Plant Soil 39:205–207

Bohnert HJ, Gong Q, Li P, Ma S (2006) Unraveling abiotic stress tolerance mechanisms–getting genomics going. Curr Opin Plant Biol 9:180–188

Bolouri-Moghaddam MR, Le Roy K, Xiang L, Rolland F, Van den Ende W (2010) Sugar signalling and antioxidant network connections in plant cells. FEBS J 277:2022–2037

Bors W, Heller W, Michel C, Saran M (1990) Radical chemistry of flavonoid antioxidants. In: Emerit I (ed) Antioxidants in therapy and preventive medicine. Springer, New York, pp 165–170

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254

Bray HG, Thorpe WV (1954) Analysis of phenolic compounds of interest in metabolism. Methods Biochem Anal 1:27–52

Camejo D, Martí MC, Olmos E, Torres W, Sevilla F, Jiménez A (2012) Oligogalacturonides stimulate antioxidant system in alfalfa roots. Biol Plant 56:537–544

Cavalcanti FR, Resende MLV, Carvalho CPS, Silveira JAG, Oliveira JTA (2007) An aqueous suspension of *Crinipellis perniciosa* mycelium activates tomato defence responses against *Xanthomonas vesicatoria*. Crop Prot 26:729–738

Daie J, Patrick JW (1988) Mechanism of drought-induced alterations in assimilate partitioning and transport in crops. Crit Rev Plant Sci 7:117–137

Farooq M, Wahid A, Kobayashi N, Fujita D, Basra SMA (2009) Plant drought stress: effects, mechanisms and management. In: Lichtfouse E, Navarrete M, Debaeke P, Véronique S, Alberola C (eds) Sustainable agriculture. Springer, Dordrecht, pp 153–188

Flexas J, Ribas-Carbo MIQUEL, Diaz-Espejo ANTONIO, Galm ESJ, Medrano H (2008) Mesophyll conductance to CO_2: current knowledge and future prospects. Plant Cell Environ 31:602–621

Foyer C, Rowell J, Walker D (1983) Measurement of the ascorbate content of spinach leaf protoplasts and chloroplasts during illumination. Planta 157:239–244

Gauillard F, Richardforget F, Nicolas J (1993) New spectrophotometric assay for polyphenol oxidase activity. Anal Biochem 215:59–65

Gill SS, Tuteja N (2010) Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. Plant Physiol Biochem 48:909–930

Grace SC, Logan BA (2000) Energy dissipation and radical scavenging by the plant phenylpropanoid pathway. Philos Trans R Soc Lond B Biol Sci 355:1499–1510

Han Y, Mhamdi A, Chaouch S, Noctor G (2013) Regulation of basal and oxidative stress-triggered jasmonic acid-related gene expression by glutathione. Plant Cell Environ 36:1135–1146

Hare PD, Cress WA, Van Staden J (1998) Dissecting the roles of osmolyte accumulation during stress. Plant Cell Environ 21:535–553

Hasanuzzaman M, Hossain MA, da Silva JAT, Fujita M (2012) Plant response and tolerance to abiotic oxidative stress: antioxidant defense is a key factor. In: Bandi V, Shanker AK, Shanker C, Mandapaka M (eds) Crop stress and its management: perspectives and strategies. Springer, Berlin, pp 261–315

Hayat R, Ali S, Amara U, Khalid R, Ahmed I (2010) Soil beneficial bacteria and their role in plant growth promotion: a review. Ann Microbiol 60:579–598

Hiscox JT, Israelstam GF (1979) A method for the extraction of chlorophyll from leaf tissue without maceration. Can J Bot 57:1332–1334

Hodges DM, DeLong JM, Forney CF, Prange RK (1999) Improving the thiobarbituric acid-reactive-substances assay for estimating lipid peroxidation in plant tissues containing anthocyanin and other interfering compounds. Planta 207:604–611

Horemans N, Foyer CH, Asard H (2000) Transport and action of ascorbate at the plant plasma membrane. Trends Plant Sci 5:263–267

Kocsy G, Brunner M, Ruegsegger A, Stamp P, Brunold C (1996) Glutathione synthesis in maize genotypes with different sensitivities to chilling. Planta 198:365–370

Luck H (1974) Methods of enzymatic analysis. Academic, New York, pp. 885–894

Marin Velazquez JA, Andreu Puyal P, Carrasco A, Arbeloa Matute A (2010) Determination of proline concentration, an abiotic stress marker, in root exudates of excised root cultures of fruit tree rootstocks under salt stress. Rev Rég Arides 24:722–727

Marklund S, Marklund G (1974) Involvement of the superoxide anion radical in the autoxidation of pyrogallol and a convenient assay for superoxide dismutase. Eur J Biochem 47:469–474

Moron MS, Depierre JW, Mannervik B (1979) Levels of glutathione, glutathione reductase and glutathione S-transferase activities in rat lung and liver. Biochim Biophys Acta Gen Subj 582:67–78

Noctor G, Foyer CH (1998) Ascorbate and glutathione: keeping active oxygen under control. Annu Rev Plant Biol 49:249–279

Noctor G, Veljovic-jovanovic SONJA, Driscoll S, Novitskaya L, Foyer CH (2002) Drought and oxidative load in the leaves of C3 plants: a predominant role for photorespiration? Ann Bot 89:841–850

Ordonez AAL, Gomez JD, Vattuone MA (2006) Antioxidant activities of *Sechium edule* (Jacq.) Swartz extracts. Food Chem 97:452–458

Paknejad F, Majidi HE, Nourmohammadi G, Siadat A, Vazan S (2006) Effects of drought stress on chlorophyll fluorescence parameters, chlorophyll content and grain yield in some wheat cultivars. Iran J Agric Sci 37:481–492

Pang CH, Wang BS (2008) Oxidative stress and salt tolerance in plants. In: Lüttge U, Beyschlag W, Murata J (eds) Progress in botany. Springer, Berlin, pp 231–245

Paradiso A, Berardino R, de Pinto MC, di Toppi LS, Storelli MM, Tommasi F, De Gara L (2008) Increase in ascorbate–glutathione metabolism as local and precocious systemic responses induced by cadmium in durum wheat plants. Plant Cell Physiol 49:362–374

Peeva V, Cornic G (2009) Leaf photosynthesis of *Haberlea rhodopensis* before and during drought. Environ Exp Bot 65:310–318

Potters G, De Gara L, Asard H, Horemans N (2002) Ascorbate and glutathione: guardians of the cell cycle, partners in crime? Plant Physiol Biochem 40:537–548

Ramanjulu S, Bartels D (2002) Drought-and desiccation-induced modulation of gene expression in plants. Plant Cell Environ 25:141–151

Saito K, Yonekura-Sakakibara K, Nakabayashi R, Higashi Y, Yamazaki M, Tohge T, Fernie AR (2013) The flavonoid biosynthetic pathway in Arabidopsis: structural and genetic diversity. Plant Physiol Biochem 72:21–34

Sawhney V, Singh DP (2002) Effect of chemical desiccation at the post-anthesis stage on some physiological and biochemical changes in the flag leaf of contrasting wheat genotypes. Field Crop Res 77:1–6

Scandalios JG (2005) Oxidative stress: molecular perception and transduction of signals triggering antioxidant gene defenses. Braz J Med Biol Res 38:995–1014

Sheng M, Tang M, Chen H, Yang B, Zhang F, Huang Y (2008) Influence of arbuscular mycorrhizae on photosynthesis and water status of maize plants under salt stress. Mycorrhiza 18:287–296

Tanaka A, Christensen MJ, Takemoto D, Park P, Scott B (2006) Reactive oxygen species play a role in regulating a fungus–perennial ryegrass mutualistic interaction. Plant Cell 18:1052–1066

Tausz M, Wonisch A, Peters J, Jimenez MS, Morales D, Grill D (2001) Short-term changes in free radical scavengers and chloroplast pigments in *Pinus canariensis* needles as affected by mild drought stress. J Plant Physiol 158:213–219

Wu QS, Xia RX (2006) Arbuscular mycorrhizal fungi influence growth, osmotic adjustment and photosynthesis of citrus under well-watered and water stress conditions. J Plant Physiol 163:417–425

Wu QS, Srivastava AK, Zou YN (2013) AMF-induced tolerance to drought stress in citrus: a review. Sci Hortic 164:77–87

Zhang S, Lu S, Xu X, Korpelainen H, Li C (2010) Changes in antioxidant enzyme activities and isozyme profiles in leaves of male and female *Populus cathayana* infected with Melampsora larici-populina. Tree Physiol 30:116–128

Chapter 27
Conservation Strategies of New Fungi Samples in Culture Collections: *Piriformospora indica* Case

V. Caleza, S. Castillo, M.D. Gomis, S. Kamah, R. López, D. Garcia-Seco, A. Varma, and K. Akdi

Abstract Conservation and maintenance strategies for microorganisms are essential to develop the biotechnology and microbiology of twenty-first century. It is necessary to develop methodologies to keep stable and safely each strain isolated or developed in a laboratory and to be able to keep the microbes exactly in the same conditions that they were found or selected. In the case of fungi, especially in non-sporulated, conservation and maintenance are more complicated than other microbes, due to the difficulty of lyophilizing it. Other times, it is necessary to consider that there are laboratories where it is not possible to lyophilize the samples or keep them in safety freezer due to the lack of facilities. That is why here we presented a simply methodology to find the best conservation method for a new fungus, according to the conditions of laboratories with different budgets and facilities. Along the chapter, a methodology to be followed by a researcher who has to find the more suitable method to conserve new fungus, adapting the method to the fungi characteristic and laboratory restrictions, is presented. *Piriformospora indica*, a recently discovered and promising fungus from the point of view of plant–microbe interaction biotechnology, is taken as a successful conservation case.

27.1 Introduction

One of the keystones of twenty-first century biotechnology is microbiology; nowadays, it is applied to almost every area of our lives, from agriculture to medicine. Indeed, the vast majority of living organisms in the planet are microbes, in both diversity and number (Hug et al. 2016). The complete information about the amount of microbes that inhabit our planet is still unknown, but it might be almost infinite (García-Villaraco et al. 2013). It has been shown that a single environmental sample

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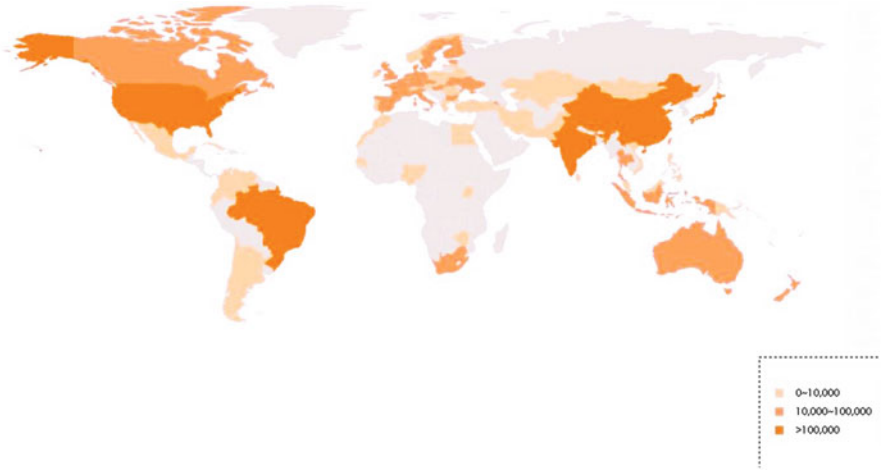

Fig. 27.1 Distribution map of preserved items in different countries and regions. The three different colors from shallow to deep on this map show the corresponding number of preserved cultures: 0–10,000; 10,000–100,000; above 100,000 items

of DNA from marine water encodes more than 1.2 million previously unknown genes from 1800 predicted genomic species (Venter et al. 2004).

The use of non-properly conserved biological material is a real problem in research and industrial processes. Well-established collections and conservation methods are necessary to provide strains and services of high quality (Homolka 2013).

Today, there is a strong worldwide interest to search and conserve the information stored in the microbial samples and individual microbe strains. The first recorded service culture collection was the Král Collection established in 1890 at the German University of Prague, Czech Republic, where cultures of microbes were made commercially available (Sette et al. 2013). However, the first independent center to endeavor to preserve and supply a wide range of microbes was the Centraalbureau voor Schimmel cultures (The Netherlands CBS), established in 1904 (Hawksworth 2004). There are 705 Culture Collections around the world, distributed in 72 countries, with 2.5 million microorganisms (among them, 1 million Bacteria, 0.7 million Fungi, and 37,787 Virus) (Juncai et al. 2014). Also, it is possible to observe that the collections are widely distributed around the world (Fig. 27.1).

The increase of the registered cultures in the World Federation for Culture Collections shows the increasing interest in this topic (Fig. 27.2). Hence, this evolution confirms the importance to develop new strategies to optimize microbe conservation techniques (Homolka 2013).

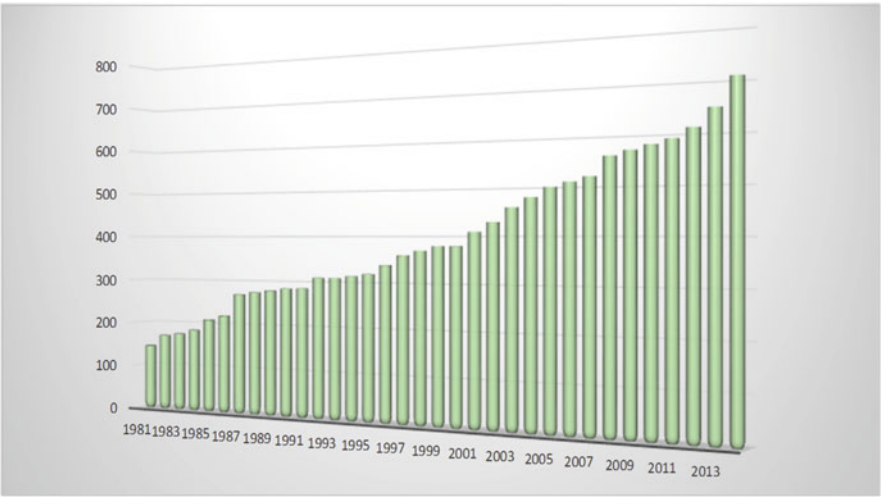

Fig. 27.2 Culture collection registered until 2014 (Juncai et al. 2014)

27.2 Classification of Microbes Conservation Methods

The correct conservation of the strain should be done by keeping it alive, pure, and genetically stable. There are a lot of conservation methods classified into two main groups: hypobiotic state (where the cells are reduced to minimum vital activity) and anabiotic state (where the growth of cells has been stopped by freezing or freeze-drying):

1. **Anabiotic state**: are the most suitable methods. The risk of contamination or alteration of the strain is minimal; however, not all the strains are compatible with these methods.
   (a) **Cryopreservation or freezing**: Cell growth is standby because the free water is frozen, but cells are alive. In this method, the temperature should be under −40 °C, but it is more convenient around −80 °C or lower in the case of liquid nitrogen. In addition, cryoprotectant agents should be used to protect cells from damage during freezing.
   (b) **Lyophilizing or freeze-drying**: Cell growth is standby because the cells have been dehydrated softly by sublimation. It is a process in which water is frozen, followed by its removal from the sample, initially by sublimation (primary drying) and then by desorption (secondary drying). Likewise, cryoprotectant agents should be used to protect cells during freezing.
2. **Hypobiotic state**: there are several strains complicated to conserve following the methods described above (like genus *Spirillum* or *Rhodospirillum*). In these cases, several methods have been developed:

(c) **Periodic transference or subcultivation**: This is a very simple and low-cost technique that allows subcultures live for a longer time, reaching to a survival of 2–30 years depending on the strain. Although some strains can adapt well the subsequent transfer, the drawback of this method is that, because the cells are alive, genetic, morphological, and physiological changes are quite common (without forgetting the risk of contamination in each subcultivation) (Castellani 1939).

(d) **Mineral oils**: The method essence is covering the well-grown culture on liquid or agar nutrient medium with sterile nontoxic mineral oil. The most common oil used is paraffin with layer thickness 1–2 cm. The aim is to limit the oxygen access that reduces the microorganisms' metabolism and growth, as well as to restrict the cell drying during preservation in freezing conditions. Following this method the microbes conservation is possible without subcultivation up to 12 years (Uzunova-Doneva & Donev 2005; Lima Freire et al. 2016).

(e) **Water or water-salt solutions**: The cells are placed in liquid medium with a compound to avoid the osmotic shock. Although this method is recommended for short-term storage at 4–8 °C for 1 week to 12 months, it can be used for some fungi, where it is demonstrated that 80% of strains survived up to 20 years at room temperature (Bueno & Gallardo 1998).

(f) **Drying**: The cultures are mixed with carriers as sterile soil, mud, active carbon, sawdust, synthetic balls, or polymers. This method takes advantage of the natural properties of the microbes to survive in dry environments until the conditions are appropriate.

The last two methods are not usually used for big collections, but they are commonly used for agro-biotechnology companies to store the biofertilizers or biopesticides with high shelf life.

27.3 Conservation of Fungi Collections

Although the properties of fungi have been exploited for thousands of years, mainly in brewing and baking, with the last years "biotechnology boom," the interest in fungi has increased, resulting in the introduction of the term "myco-technology" (Bennett 1998). Nowadays, fungal strains can be applied to many social-economic areas, including the production of a wide range of commercially interesting compounds, such as biofertilizers, biostimulants or biopesticides, enzymes, antibiotics, pigments, vitamins, alcohols, organic acids, pharmaceuticals, cosmetics, among others. The main issue to develop and apply these fungi is the problem related with strain conservation.

There is a rich literature on the preservation of fungal cultures, and this chapter will not review all possible techniques. The aim of this chapter is to design a protocol to conserve a new fungus for which conservation method has not been optimized yet (Fig. 27.3).

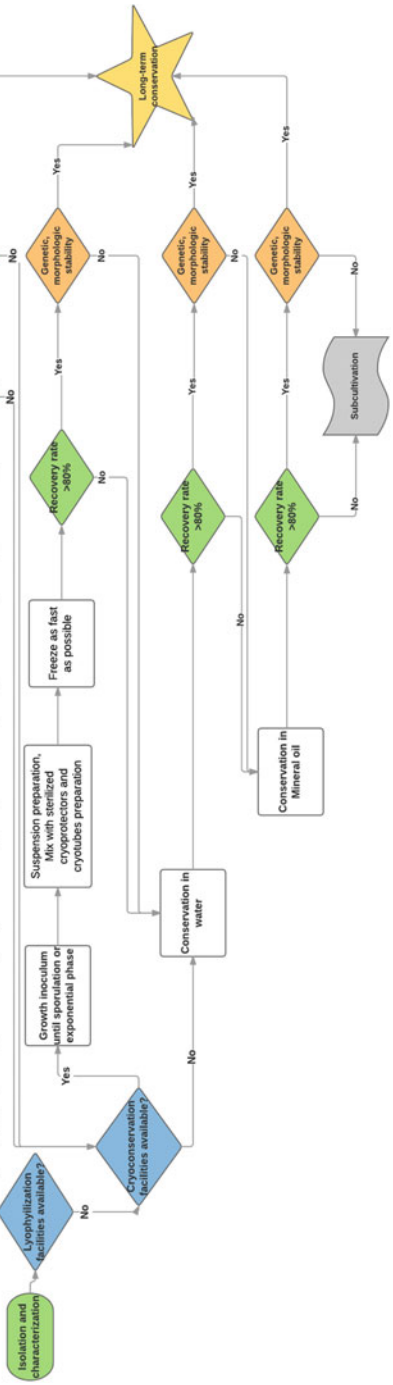

Fig. 27.3 Flowchart of proposed methodology to find the best conservation method

27.3.1 Lyophilization

The more suitable way for long-term conservation of fungi is lyophilization (Fig. 27.4). This method should be the first to check if the laboratory has the needed facilities. This method has several benefits; cultures can be stored in little space at room temperature during long-term conservation and are easily sent elsewhere. It is effective for a lot of conidial fungi as ascomycetes and basidiomycetes. It is not usually useful for fungi with very "watery" cells with large vacuolar volumes but may succeed if the spores are not heavily vacuolated (e.g., the sporangiospores of many zygomycete fungi). Also, it has to be considered that, in general, spores can tolerate the most of conservation methods better than vegetative hyphae, and lyophilizing is not the exception. Large-scale processes use bottles dried on shelves in a large vacuum chamber and sealed under vacuum by the lowering of a pressure plate onto the partially seated rubber stoppers (Humber 2012).

There are two main problems associated with this method: first of all, the availability of the lyophilizer. The second issue is that there are a lot of fungi that it is not possible to recover, especially the ones that cannot sporulate (Homolka

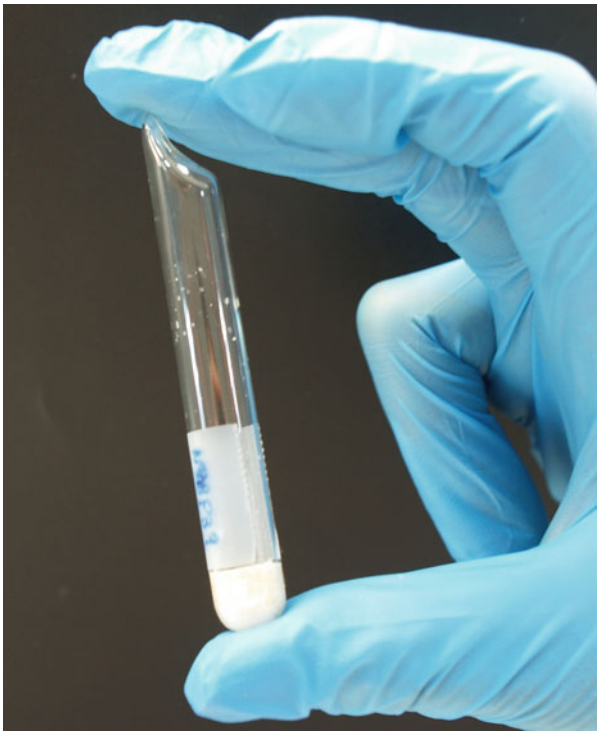

Fig. 27.4 Example of lyophilized sample

2013). For example, in our lab we had problems in conservation by lyophilization of several fungi, so, we had to utilize cryopreservation techniques that after several tests worked perfectly in long-term conservation.

In these cases, we can choose two kinds of methods depending on the conditions of the lab: if a −80 °C freezer or a liquid nitrogen freezer is available, freezing is the most recommended method. In the case it is not available or the electricity is not assured, we should try "low-cost" methods, like Mineral oil or Water or water-salt solutions.

27.3.2 Freezing

It was introduced in 1960 in ATCC (American Type Culture Collection) for long-term storage of large numbers of fungal species with very good results (Homolka 2013; Hwang 1960). Nowadays, it is the main conservation technique in the world, with lyophilizing.

Agar block with the fungal mycelium and spores immersed in an appropriate cryoprotectant is the traditional and simplest method. However, other methods exist as polystyrene beads or porous ceramic beads (Chandler 1994; Palagyi et al. 1997).

The two main factors are freezing and thawing conditions. In general, there are two possibilities: slow or controlled freezing or fast, with pros and cos in both of them. Although each microbe is different, generally too low freezing rates cause extreme dehydration and concentration of the solution, leading to cell damage. On the other hand, too fast freezing causes insufficient dehydration and formation of abundant ice crystals.

Other important factor is the cryoprotectant applied. Protective compounds or cryoprotectants are found to eliminate most of the multiple destructive factors during freezing of biological structures. The main cryoprotectants are dimethylsulfoxide (Me_2SO), glycerol, blood serum or serum albumin, skimmed milk, yeast extract, saccharose, glucose, methanol, peptone, polyvinylpyrrolidone (PVP), sorbitol, and malt extract.

Cryoprotectants can be classified in various ways, such as either low-MW or high-MW (Molecular Weight) additives or depending on the rate of penetration (Hubalek 2003); those that penetrate quickly, around 30 min, include methanol, ethanol, ethylene glycol (EG),1 propylene glycol (PG), dimethylformamide, methylacetamide, and Me_2SO. Also, Glycerol is among the most important ones, which penetrates more slowly. Nonpenetrating or nonpermeating are polyvinylpyrrolidone (PVP), polyethylene glycol (PEG), polyethylene oxide (PEO), or polyvinyl alcohol, mono-, oligo-, and polysaccharides, mannitol, sorbitol, dextran, hydroxyethyl starch (HES), methyl cellulose, albumin, and gelatin.

The most usual cryoprotectants are Me_2SO and glycerol. Dimethylsulfoxide was firstly used to cryoprotect red blood cells (RBC) and spermatozoa (Lovelock &

Bishop 1959). The optimum Me_2SO concentration varies widely, from 1 to 32% (median around 10%). *Neurospora crassa*, *Sclerospora sorghi*, certain *Pezizales*, *Volvariella volvacea*, and other basidiomycetes have been conserved with dimethylsulfoxide (Hubalek 2003; Barnhart & Terry 1971; Challen & Elliot 1986; Homolka 2013). Many fungi do not usually tolerate high concentrations of Me_2SO, but no marked toxicity of Me_2SO to filamentous fungi has been described (Hubalek 2003). Among alcohols, Glycerol (1,2,3-propanetriol) and Polyethylene glycol (PEG) have been the most widely used in fungi. Also, glycerol allows intermediate methods between freezing and room temperature conditions. For example, *Aspergillus*, *Penicillium*, or *Trichoderma* have been conserved up to 30 months, in glycerol 50% (Shankar Paul et al. 2015).

27.3.3 Water or Water–Salt Solutions

Sometimes, lyophilization or freezing is not possible because of the fungi or the lab conditions. It was demonstrated that conservation under pure sterile water or water-salt condition can be a cheap alternative and low space-demanding technique. Cells are placed in liquid medium, and they approach a hypobiotic state. The suspension density, the presence of Ca^{2+} ions in the medium, the solution composition, pH, and the preservation temperature influence the quantity and protection of the cells. Some fungi can endure up to 20 years in sterile water (De Capriles et al. 1989), but the danger is that some fungi lose viability much sooner. In fact, distilled water was the most appropriate form of preservation of endophytic microorganisms in recent published work (Lima Freire et al. 2016). Some problems for this technique are easily avoided: too much inoculum for the volume of water may threaten the ability of the fungus to withstand long-term storage (the water volume should be around 40 times greater than the inoculum blocks) (Humber 2012). Too much medium in the vial can contain too much nutrients that shorten the longevity of the stored fungus.

27.3.4 Mineral Oil

Storing culture under a layer of sterile mineral oil is an approach still widely used when the methods above are not possible. The oil prevents dehydration as well as it reduces gas exchange, reducing fungal metabolism to a very low level. If there are spaces available to store racks of tubes, this is a common alternative for the storage. Cultures under mineral oil may remain viable for decades (Silva et al. 1991; Dasilva et al. 1994).

A comparative study was conducted on long-term fungal culture preservation methods. A total of 112 isolates of several phytopathogenic fungi (*Alternaria alternata*, *A. solani*, *Fusarium oxysporum* f. sp. *lentis*, *F. oxysporum* f. sp. *capsici*,

Rhizoctonia solani, *Lasiodiplodia theobromae*, *Colletotrichum gloeosporioides*, and *Curvularia lunata*) were stored up to 2 years by using five preservation methods. Although the stability and regeneration rate of mineral oil method was not the best, it produced goods results. In all cases, there was more than 50% of percentage of revival, so, it could be suitable in some lab conditions (Aliya et al. 2015).

27.4 Example of a Problematic Case: Conservation of *Piriformospora*

Piriformospora indica (a mycorrhiza like endophytic Agaricomycetes fungi) has received great attention over the last few decades, due to its exceptional ability to efficiently promote plant growth, protection, and stress tolerance (Gill et al. 2016; Varma et al. 1999; Varma and Kost 2013). Due to the possibility of *P. indica* to be cultivable axenically and its versatility for colonizing/hosting a broad range of plant species, it has a great potential for biotechnological applications (Fig. 27.5).

In fact, in 2016 a molecular ecology study revealed the hyperdiversity of Sebacinales and their evolutionary diversification into two sister families, *Sebacinaceae* (that forming basidiomes, isolation into pure culture so far only for early-diverging saprotrophic species, and endophytic, ectomycorrhizal, and orchid mycorrhizal in partially or fully mycoheterotrophic plants) and Serendipitaceae (basidiomes never observed until now, frequently isolated into pure cultures from orchid roots, frequently endophytic, orchid mycorrhizal in green species, ericoid mycorrhizal, symbiotic of liverworts, and ectomycorrhizal in some lineages). And it is proposed to transfer the endophytic cultivable species *P. indica* and *P. williamsii* to the genus *Serendipita* (Weiß et al. 2016).

Fig. 27.5 *Piriformospora indica* growing in liquid medium

Because of the interest in developing biofertilizers and biostimulants based on *Piriformospora*, it is vital to develop a method that can ensure the conservation of the strain without any single genetic or morphologic modification.

One of the first problems is that *Piriformospora* spp. loses its root colonization efficiency (or endophytic capacity) after repeated subculturing on synthetic medium. To maintain this efficiency, the fungus must be periodically inoculated to the roots of host plants (in vitro or in soil) and re-isolated from the internally colonized roots (Schedel et al. 2012; Johnson et al. 2011). Therefore, it was really important to find an anabiosis method to conserve the strain stable and in perfect conditions.

As it is explained above, the first candidate technique for conservation was lyophilizing. We tried it and studied the regeneration rate and stability after several time points:

1. Culture preparation: Because this method requires fungi spores, once the inoculum was sporulated (Fig. 27.6), cultures were mixed with sterilized skim milk solution (20%). Others media are serum, peptone, various sugars etc.
2. In parallel, ampoules were closed with small cotton plugs and autoclaved.
3. Then, the mix was aliquoted in the sterilized ampoules. The preparations were frozen in −80 °C freezer.
4. Once frozen, the ampoules were inserted in a strong vacuum to the lyophilizer (Fig. 27.7).
5. As soon as it is lyophilized, it is necessary to check that the sample is completely dried.
6. Ampoules were flame-sealed while it is still under vacuum.
7. Conservation. Although the lyophilized samples can be kept at room temperature, it is recommended to keep at 4 °C.

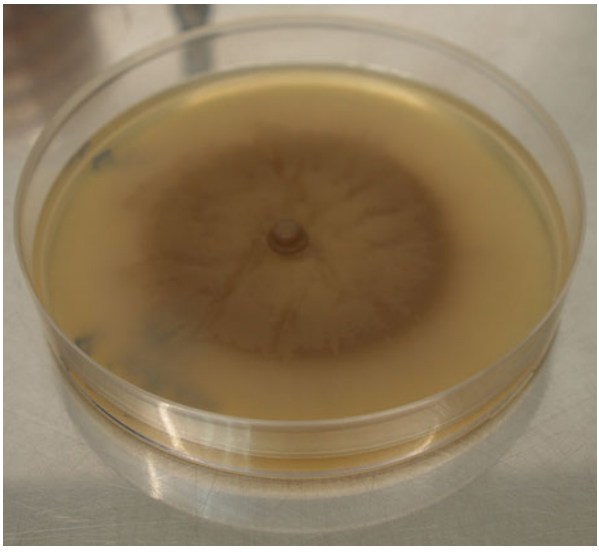

Fig. 27.6 *Piriformospora indica* growing in solid medium

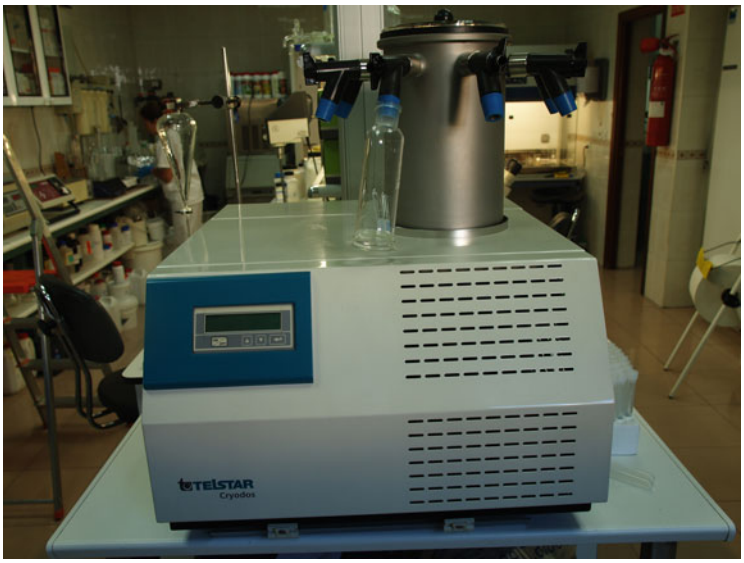

Fig. 27.7 Typical laboratory lyophilizer

8. Monitoring. To study the stability and recovery rate, the recovery of the culture was analyzed each month during 2 years. To recover the culture, it was necessary just to sterilize the surface of the ampoule with ethanol or similar, open it scoring the neck, and add sterile water or liquid medium to the freeze-dried contents to reconstitute the culture.

The results of this study were not satisfactory because the recovery rate were under 10% after 6 months, which is far from being adequate for a long-term collection.

As noted above, we tried the second method, the cryo-conservation. We chose it because conserving the samples below −80 °C will ensure their long-term integrity.

Cryo-conservation protocol:

1. Culture preparation: it is better to use the spores mixed with hyphae. If it is not possible, we should use the culture in the exponential growth phase.
2. Suspension preparation: If the culture is liquid, it should be centrifuged gently to remove part of the nutrients and concentrate it, increasing the number of cells in the final preparation. It is necessary to check the total and viable unit colonies forming. Then, it is necessary to use a protective media to keep the microorganism viability that the researcher has to choose among some of the listed above (it has to be chosen empirically). It must be nontoxic, have good water solubility and low eutectic temperature, prevent salt hyperconcentration in the suspension, stabilize hydrogen connections in the crystal lattice, and prevent large crystal

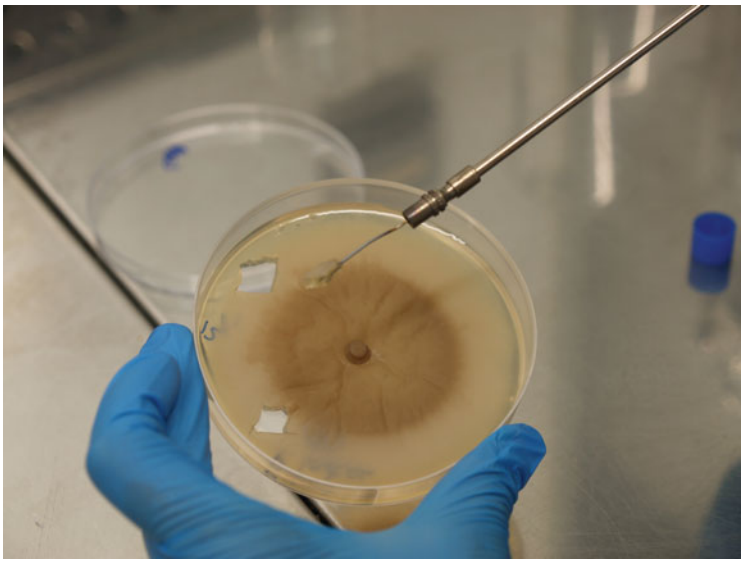

Fig. 27.8 Cutting the cubes of agar with *Piriformospora indica* to cryopreservation

forming. In a new fungus always it is appropriate to check several cryoprotectors. After several tests, we decided to use glycerol to conserve *P. indica* (Fig. 27.8). The common method is to apply 500 µl glycerol 30% to 500 µl of the culture. However, we checked diverse methods, concluding that the best is to add 4 cubes of 0.5 cm × 0.5 cm approx. of agarized medium with the *P. indica* to a dilution of glycerol 10% (Fig. 27.9).

3. Cryotubes preparation: Once best conservant is decided, it is necessary to autoclave the cryotubes containing 1 ml of glycerol 10%.
4. Freeze as fast as possible. If it is possible, sink in liquid nitrogen before to keep in the −80 °C freezer or preserve directly in 80 °C freezer.
5. Monitoring: Always it is recommended to check the stability of the culture periodically to ensure a long-term conservation of a new fungus. Our results (Fig. 27.10) show a monthly monitoring. We found a recovery rate close to 100% after 2 years.

When we are working with fungi that interact with other organisms, it is very recommendable to check that it is keeping this capacity of interaction. In our study, after 2 years of cryoconservation (as it was explained in the protocol above), we tested the root colonization efficiency of *P. indica* under controlled conditions. The cocultivation experiments were carried out with the model plant *Arabidopsis thaliana* in Petri dishes and with *Solanum lycopersicum* plants in growth chamber).

For *Arabidopsis thaliana* assay, cocultivation of *Piriformospora* was done as described by JM Johnson et al. (2011), with modifications. Colonized and uncolonized roots were cut after 27 and 20 days of cocultivation. The roots were

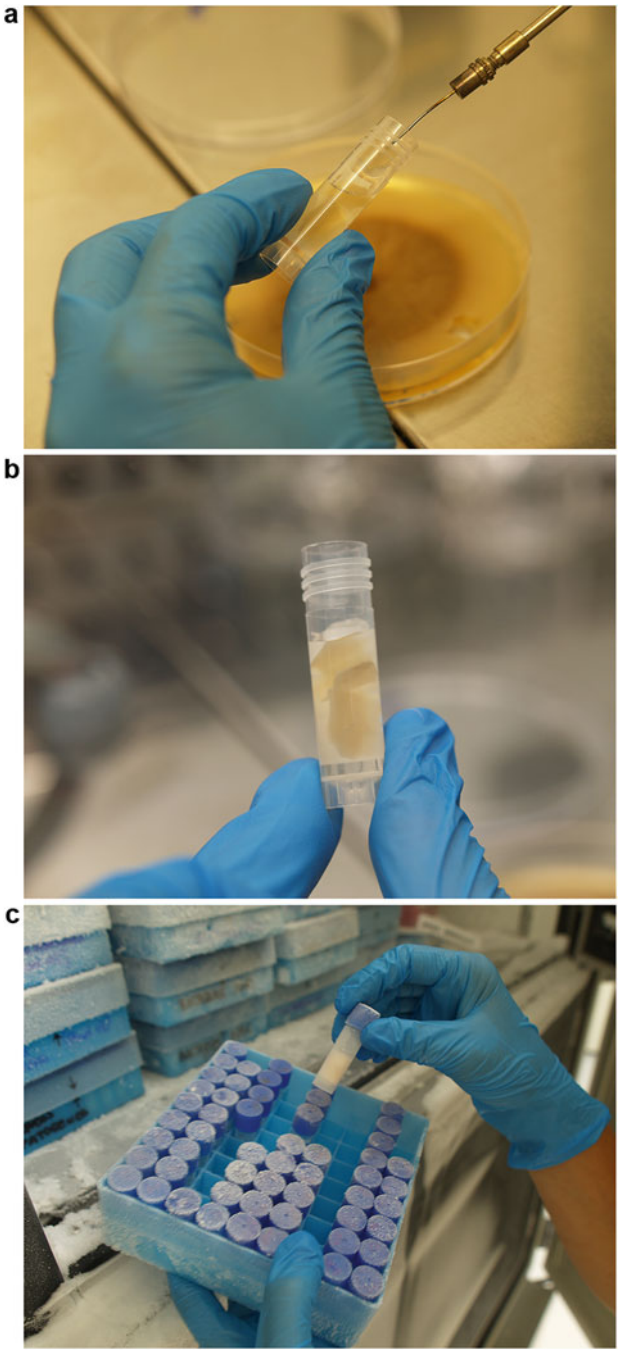

Fig. 27.9 Procedure to keep the samples by cryopreservation. (**a**) Immersion of the agar cubes in cryoprotectors. (**b**) Cubes in cryotubes. (**c**) Cryotubes stored in −80 °C

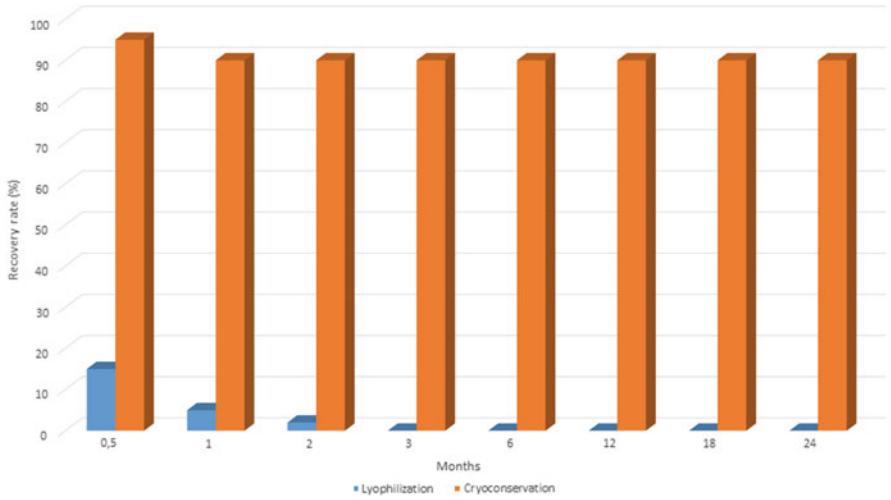

Fig. 27.10 Recovery rate of *P. indica* in the two methods tested along 2 years monitoring it

treated with 10% KOH and incubated overnight at room temperature in Petri dishes. After incubation, roots were water washed and incubated in 1% HCl for 3 min. Finally, the roots were washed again and dyed using lactophenol blue solution, in order to observe the preparation under 10X objective in the microscope.

To re-isolate the fungus, roots were surface cleaned with 0.1% sodium hypochlorite for 2 min, water washed, and dried in flow chamber. In order to observe the fungus growth along the root pieces, they were placed on Hill and Kaefer plates and incubated 5–7 days at 30 °C in the dark. The fungus was then immediately subcultured by taking the hyphal tip to fresh Hill and Kaefer plates.

For the tomato bioassay (var. "Marmander"), tomato seedlings (one- to two-true-leaf stage) grown in Brown 25 W peat (Projar, Valencia, Spain) were transplanted into each pot with PS Potplant substrate (Projar, Valencia, Spain). Plants were irrigated as needed and fertilized with Hoagland nutrient solution twice per week. The assay was carried out in a growth chamber at 25 °C, photosynthetically active radiation (PAR) intensity 280 μE/m^2 s, photoperiod 16 h light and 8 h dark, and 60% relative humidity.

The endophyte *Piriformospora* was propagated on solidified Hill and Kaefer medium, at 30 °C. Fungal plugs of 5 mm diameter were made with cork borer and transferred to the flasks with 250 ml Kaefer liquid medium. The flasks were further incubated at 30 °C and 140 rpm for 2 weeks (Adya et al. 2013). Mycelium and spores were mixed with a blender for 2 min at lowest speed in sterile distilled water. Chlamydospore total number was examined in a Thoma chamber, and the number of viable propagules was determined by plating on solid Hill and Kaefer media (Fakhro et al. 2010). The suspensions were adjusted with sterile distilled water to a

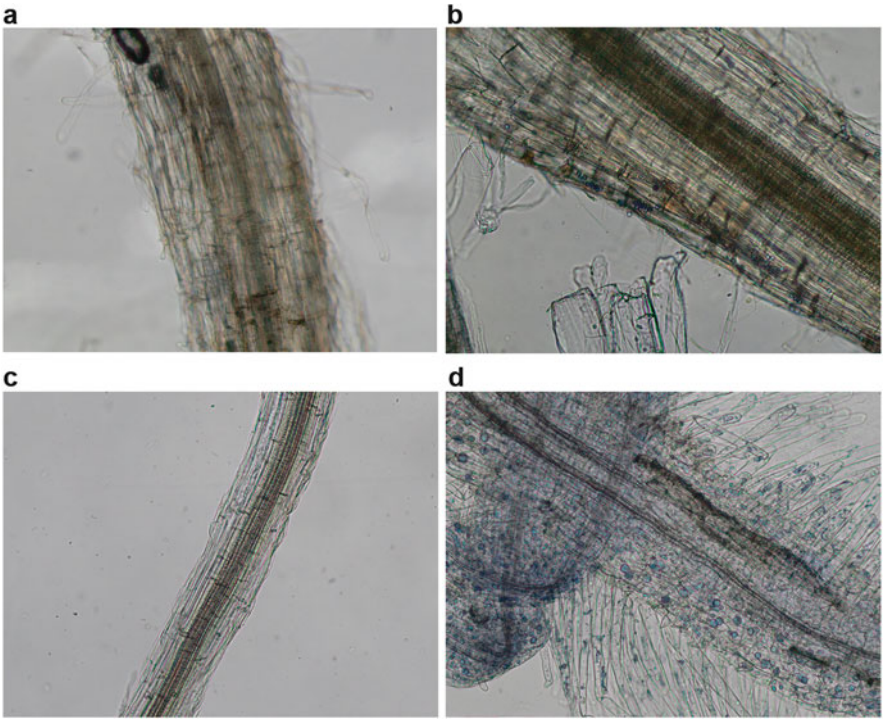

Fig. 27.11 *Piriformospora indica* in a root colonization efficiency test, in tomato ((**a**) *Solanum lycopersicum* root control; (**b**) *S. lycopersicum* root inoculated with *P. indica;* (**c**) *Arabidopsis thaliana* root control; (**d**) *A. thaliana* root inoculated with *P. indica*)

concentration of 10^4 CFU/ml, not exceeding 5×10^4 CFU per plants (Fakhro et al. 2010).

Piriformospora was applied to the tomato plants at 7, 14, and 21 days post-transplant through irrigation. The assays were established with twelve plants per treatment, distributed in four randomized blocks.

At the end of the bioassay, to check *Piriformospora* "root colonization efficiency" persistence, the roots were prepared as explained above (Johnson et al. 2011) and examined under the microscope (Fig. 27.11).

27.5 Concluding Remarks

The conservation strategies are one of the twenty-first century biotechnology keystones. Traditional subculture methods to keep alive fungus are not desirable in the "new generations" biotechnology. When these traditional methods are long-therm applied, they can trigger mutations and unnatural selections. It is essential to be able to keep the fungi exactly in the same conditions that it was isolated or

selected. Sometimes, it is difficult to find the best method to conserve fungi, especially with non-sporulated fungus, that are not possible to keep them lyophilized, and it is necessary to search alternative methods to keep them stable. In this chapter, we present a simple methodology to find the best conservation method for a new fungus, according to the conditions of laboratories with different budgets and facilities.

References

Adya A, Gautam A, Zhang L, Varma A (2013) Characterization of *Piriformospora indica* culture filtrate. In: Varma A, Kost G, Oelmüller R (eds) *Piriformospora indica*. Springer, Berlin, pp 345–375

Aliya T, Naz F, Rauf CA, Irshad G (2015) Long term and least expensive preservation methods for various fungal cultures. Pak J Phytopathol 27:147–151

Barnhart EE, Terry CE (1971) Cryobiology of *Neurospora crassa*. Cryobiology 8:323–327. doi:10.1016/0011-2240(71)90125-8

Bennett JW (1998) Mycotechnology: the role of fungi in biotechnology. J Biotechnol 66:101–107

Bueno L, Gallardo R (1998) Filamentous fungi preservation in distilled water. Rev Iberoam Micol 15:166–168

Castellani A (1939) The viability of some pathogenic fungi in sterile distilled water. J Trop Med Hyg 42:225–226

Challen MP, Elliot TJ (1986) Polypropylene straw ampoules for the storage of microorganisms in liquid nitrogen. J Microbiol Methods 5:11–22

Chandler D (1994) Cryopreservation of fungal spores using porous beads. Mycol Res 98:525–526

Dasilva AMM, Borba CM, Deoliveira PC (1994) Viability and morphological alterations of *Paracoccidioides brasiliensis* strains preserved under mineral oil for long periods of time. Mycoses 37:165–169

De Capriles CH, Mata S, Middelveen M (1989) Preservation of fungi in water (Castellani): 20 years. Mycopathologia 106:73–79

Fakhro A, Andrade-Linares DR, von Bargen S et al (2010) Impact of *Piriformospora indica* on tomato growth and on interaction with fungal and viral pathogens. Mycorrhiza 20:191–200

García-Villaraco A, Bonilla A, Garcia-Seco D, Algar E (2013) Metagenomics of plant-microorganism interaction: source of novel recombinant genes for biotechnological application. In: Rodelas B (ed) Beneficial plant-microbial interactions: ecology and applications. CRC Press, Hoboken

Gill SS, Gill R, Trivedi DK, Anjum NA, Sharma KK, Ansari MW, Ansari AA, Johri AK, Prasad R, Pereira E, Varma A, Tuteja N (2016) *Piriformospora indica*: potential and significance in plant stress tolerance. Front Microbiol 7:332. doi:10.3389/fmicb.2016.00332

Hawksworth DL (2004) Fungal diversity and its implications for genetic resource collections. Stud Mycol:9–17

Homolka L (2013) Methods of cryopreservation in fungi. In: Gupta VK et al (eds) Laboratory protocols in fungal biology: current methods in fungal biology. Springer, New York, pp 9–15

Hubalek Z (2003) Protectants used in the cryopreservation of microorganisms. Cryobiology 46:205–229

Hug LA, Baker BJ, Anantharaman K et al (2016) A new view of the tree of life. Nat Microbiol 1:16048. doi:10.1038/nmicrobiol.2016.48

Humber RA (2012) Preservation of entomopathogenic fungal cultures. In: Manual of techniques in invertebrate pathology. pp 317–328

Hwang S-W (1960) Effects of ultra-low temperatures on the viability of selected fungus strains. Mycologia 52(3):527. doi:10.2307/3755974

Johnson JM, Sherameti I, Ludwig A, Nongbri P, Sun C, Lou B, Varma A (2011) Protocols for *Arabidopsis thaliana* and *Piriformospora indica* co-cultivation—a model system to study plant beneficial traits. J Endocytobiosis Cell Res 21:101–113

Juncai M, Hideaki S, Desmeth P (2014) World directory of culture collections (Sixth version, 2014). Database. http://www.wfcc.info/ccinfo/home/

Lima Freire AK, dos Santos BA, Lima Sampaio I, Moura de Lima A, Botineli L, Coelho da Rocha L, Braga De Souza J, Souza É (2016) Availability and morphological characteristics of endophytic fungi held in different methods of preservation. Sci Res Essays 11:76–79

Lovelock KE, Bishop MWH (1959) Prevention of freezing damage to living cells by dimethylsulphoxide. Nature 183:1394–1395

Palagyi Z, Nagy A, Vastag M, Ferenczy L, Vagvölgyi CS (1997) Maintenance of fungal strains on cryopreservative-immersed porous ceramic beads. Biotechnol Tech 11:249–250

Schedel S, Camehl I, Oelmüller R (2012) Long-term interaction between *Arabidopsis* and *Piriformospora indica* on expanded clay. Endocyt Cell Res 22:1–5

Sette LD, Pagnocca FC, Rodrigues A (2013) Microbial culture collections as pillars for promoting fungal diversity, conservation and exploitation. Fungal Genet Biol 60:2–8

Shankar Paul J, Tiwari KL, Jadhav SK (2015) Long term preservation of commercial important fungi in glycerol at 4 °C. Int J Biol Chem 9:79–85

Silva M, Lima D, Cavalcanti M (1991) Induction of the sporulation in *Colletotrichum gloeosporioides* Penz, preserved under mineral oil. Pesq Agrop Brasileira 1(11):12

Uzunova-Doneva T, Donev T (2005) Anabiosis and conservation of microorganisms. J Cult Collect 4:17–28

Varma A, Kost GOR (2013) *Piriformospora indica*—Sebacinales and their biotechnological application. Springer, Berlin. isbn: 978-3-642-33802-1

Varma A, Verma S, Sahay N (1999) *Piriformospora indica*, a cultivable plant-growth-promoting root endophyte. Appl Environ Microbiol 65:2741–2744

Venter JC, Remington K, Heidelberg JF, Halpern AL, Rusch D, Eisen J, Wu D, Paulsen I, Nelson K, Nelson W, Fouts D, Levy S, Knap A, Lomas M, Nealson K, White O, Peterson J, Hoffman J, Parsons R, Baden-Tillson H, Pfannkoch C, Rogers Y, Smith H (2004) Environmental genome shotgun sequencing of the Sargasso Sea. Science 304:66–74

Weiß M, Waller F, Zuccaro A, Selosse M-A (2016) Sebacinales—one thousand and one interactions with land plants. New Phytol 211:20–40

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