Tulasi Satyanarayana
Subrata Kumar Das
Bhavdish Narain Johri *Editors*

Microbial Diversity in Ecosystem Sustainability and Biotechnological Applications

Volume 2. Soil & Agroecosystems

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Tulasi Satyanarayana • Subrata Kumar Das
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Volume 2. Soil & Agroecosystems

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Preface

The outer loose material of the Earth's surface, which is distinctly different from underlying bedrock, is soil. This originates from physico-chemical and biological weathering of rocks. Soil is an admixture of five major components, viz. mineral matter, organic matter, soil-air, soil water and soil organisms. Soil is not a mass of debris, but teeming with life. Every small particle of soil contains numerous types of living organisms belonging to the Bacteria, Archaea and Eucaryota domains, and viruses; living organisms which are too small to be seen with the naked eye are microbes, microorganisms or microscopic organisms.

Soil microorganisms are vital for the continuing cycling of nutrients and for driving aboveground ecosystems. 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. Soil microorganisms influence aboveground ecosystems by contributing to plant nutrition, plant health, soil structure and soil fertility. The activity and species composition of microbes are, however, generally influenced by many factors including physico-chemical properties of the soil, temperature and vegetation. The dynamics of soil microbes have important implications for the response of subsurface soil ecosystems to perturbations. Despite all attempts to measure fluxes and gross microbial pools, the soil and its microbiota still remain a black box. Most soil microorganisms are still unknown. The comparison between direct microscopic counts and plate counts indicates that less than 0.1% of agricultural soil microorganisms are culturable. Understanding the diversity and dynamics of indigenous microbial populations represents a challenge to modern soil ecology.

The rhizosphere, the narrow zone of soil that is influenced by root secretions, can contain up to 10^{11} microbial cells per gram root and more than 30,000 prokaryotic species. The collective genome of this microbial community is much larger than that of the plant, which is also known as the plant's second genome. The microflora of most soils is carbon starved. Since plants secrete up to 40% of their photosynthates into the rhizosphere, the microbial population densities in the rhizosphere are much higher than those in the surrounding bulk soil; this phenomenon is known as the 'rhizosphere effect'. In general, rhizosphere microbial communities are less diverse than those in bulk soil.

The mesmerizing diversity of microbes settling down in the rhizosphere are termed "root microbiome," while their interaction with roots can result in a positive or negative outcome for plant fitness. Increasing evidence suggests that the

microbial communities dominating the rhizosphere are influenced by the host genotype. It appears that plants actively select and determine the composition of the root microbiome by releasing compounds in the rhizosphere which selectively stimulate microorganisms promoting plant growth and health or repress organisms that are deleterious to plants. Root microbiome is, therefore, a subset of a more diverse microbial community recruited from the surrounding bulk soil. The involvement of root microbiome on plant health becomes more evident in disease-suppressive soils. In these soils, a plant is unlikely to become infected by a soil-borne pathogen even when the pathogen is present and favoured by the plant. The phenomenon of disease suppression is well-known and is associated with the indigenous microbiota and activity. The mechanisms underlying this phenomenon have not, however, been fully understood. In this volume, Dubey and Sharma (Chap. 11) discuss the concept of rhizosphere engineering by employing synthetic microbial communities and the prospects of the rhizosphere microbiome engineering.

Microbial diversity represents the variability among all types of microbes (prokaryotes [archaea and bacteria], eukaryotes [algae, fungi, protozoa] and acellular viruses and others) in the natural world. Interest in the exploration of microbial diversity has stemmed from the fact that microbes are essential for life because they perform numerous functions essential for the biosphere that include nutrient cycling and environmental detoxification. The vast array of microbial activities and their importance to the biosphere and to human economies provide strong rationale for understanding their diversity, conservation and exploitation for society.

For long, microbial diversity has been explored by conventional culture-dependent methods, which allow access to only 0.1–1.0% of the extant microbes in any ecosystem. Over the past two decades, several methods, such as rRNA gene sequencing, fluorescence *in situ* hybridization (FISH), denaturing gradient gel electrophoresis (DGGE), temperature gradient gel electrophoresis (TGGE), restriction fragment length polymorphism and terminal restriction fragment length polymorphism (T-RFLP), have been developed to assess microbial diversity and catalogue microbes without the need for isolation. The use of molecular techniques over the past 20 years has shown that only a very small fraction of microbial diversity so far has been catalogued from all the habitats investigated.

Earth is considered to be inhabited by close to a trillion bacterial and archaeal species, and 10–15 million eukaryotic species; this prediction is based on ecological theory reformulated for large-scale predictions, an expansive dominance scaling law, a richness scaling relationship with empirical and theoretical support and the largest molecular surveys compiled to date [PNAS (2016) 113: 5970–5975]. The profound magnitude of our prediction of Earth's microbial diversity emphasizes the need for continued investigation.

Extensive and intensive efforts are being made to understand microbial diversity by both culture-dependent and culture-independent metagenomics approaches. Despite significant advances made in understanding microbial diversity, most microbes are characterized only by 'molecular fingerprints' and have resisted cultivation. The microbiomics approach is now being adopted for surveying total

microbes present in different ecosystems (e.g. earth microbiome, ocean microbiome, human microbiome and rhizosphere microbiome, to mention a few). In order to analyse microbial populations in ecosystems such as skin and mucosal surfaces of humans and animals, plants, soil and oceans, Next Generation Sequencing (NGS) and advanced bioinformatics have become valuable tools. The Earth Microbiome Project (EMP), launched in 2010, is a landmark study for investigating large-scale microbial diversity. Bacterial and archaeal 16S rRNA diversity in 27,751 samples was analysed from 97 independent studies, which produced 2.2 billion sequence reads [PNAS (2018) 115: 4325–4333]. Only two-thirds of EMP reads could be mapped to the existing 16S references, which prevented meaningful Operational Taxonomic Unit (OTU) analysis.

By leveraging metagenomics and metabarcoding of global top soil samples (189 sites, 7,560 subsamples), it has recently been observed that bacterial genetic diversity is very high in temperate habitats in comparison with fungi, and microbial gene composition varies more strongly with environmental variables than with geographic distance [Nature (2018) 560: 233–237]. Fungi and bacteria show global niche differentiation which is associated with contrasting diversity responses to precipitation and soil pH. Both competition and environmental filtering have been seen to affect the abundance, composition and encoded gene functions of bacterial and fungal communities, suggesting that the relative contributions of these microorganisms to global nutrient cycling varies spatially.

Good understanding of microbial diversity will allow us to cure diseases, engineer and conserve our environment, manufacture better products, grow more food, colonize other worlds, and much more. In practical and scientific terms, microorganisms give us the power to ask new questions and solve previously intractable problems.

Voluminous data have accumulated on the microbial diversity of various ecosystems. The present attempt is to briefly review the developments in understanding microbial diversity and its role in ecosystem sustainability and biotechnological applications. The second volume of the book focuses on Soil and Agroecosystems. Very comprehensive reviews are included in this volume on aspects such as microbial diversity in caves, jhum agroecosystem, metal rich mine leachates, ectomycorrhizal and VAM fungi, mangroves, Himalayan edible mushrooms, microbes producing industrially useful enzymes and several others.

We wish to thank all the contributors for readily accepting our invitation and submitting well-written chapters in their areas of specialization within the prescribed timelines. The opinions expressed by the contributors are their own. We sincerely hope and wish that the book will serve as a ready reference for students, scholars, teachers and scientists in broad areas of life sciences, microbiology and biotechnology. We wish to thank Springer Nature for publishing the book to disseminate knowledge from innumerable sources in two volumes.

New Delhi, Delhi, India — Tulasi Satyanarayana
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Part I

Soil and Agroecosystem

Cave Microbiome for Human Welfare

1

Subhro Banerjee, D. K. Jha, and S. R. Joshi

Abstract

Nature is bountiful of living biota which ranges from 3 to 50 million and one-third of global biodiversity exists in India. Since prehistoric times, humans have exploited microorganisms for their own use. The Earth's subsurface presents one of the finest promising locations to look for microbial life and the distinctive lithologies that life leaves behind. Studies on microbial diversity are hampered not only by the technical ability to assess the species numbers but also by the high heterogeneity of the environment, with its changing temporal and spatial microhabitats. Moreover, natural products are for the most part a booming source of drug leads. Regardless, their application in innovation of new drug has fallen out of favour. Not more than 10% of the planet's biodiversity has been under trial for biological property; a lot of functional natural compounds are pending innovation. The test is how to get in touch with this natural chemical diversity. In this aspect, research on caves is the utmost need of the hour to increase our acceptance of the means of biological adjustment to severe circumstances, the relations involving organisms and minerals, the function of inorganic matter in diverse dark ecosystems and the evolution and speciation of biological schemes under acute circumstances, progressing to a range of biotechnological uses. In the present chapter, a handful of the important caves around the world are

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described, together with an analysis of the potential health effects from the microbes inhabiting such ecosystems. Needless to emphasise, such type of study spanning over length and breadth of India is the urgent need of the hour, which hopefully would unravel many of the microbes of biotechnological importance. Suggestions for potential investigations are highlighted to promote going with the flow from qualitative research to additional experimentations.

Keywords
Microbes · Diversity · Human welfare · Cave · Bioprospecting · Geomicrobiology

1.1 Introduction

At present, various syndromes, the source of which are infectious agents, particularly by multi-drug-resistant species, have become the most important menace to community well-being around the globe. It is usually presumed that determining new, previously unknown microbial strains symbolises the most excellent opening to come across new bioactive compounds. Even though microorganisms are rarely discernible in natural environments, it is estimated that about half the biomass on Earth is microbial (Whitman et al. 1998). Furthermore, microbial life is widely distributed: where there is life on Earth, there is microbial life – microorganisms are found in all ecosystems. The term microbial ecology is thus used to describe the presence and contributions of microbes through their activities to the place where they are found (Das et al. 2006). Microorganisms, which have been evolving on Earth for at least 3.8 Ga (10^9years) out of their 4.6 Ga existence, have provided conditions on the planet that have made it habitable for all other species (Das et al. 2006). Coupled with the above, our awareness of soil microbial diversity is also restricted in part by our inability to culture them and the other by the lack of surveys in selected regions of the Earth. On the other hand, screening of 16S rRNA genes targeted by primers in soils, in addition to other techniques like metagenomics, has permitted an extra direct census of learning soil microbial diversity (Palmer et al. 2006). Thus, microbial diversity is one of the difficult areas of biodiversity research, and unlike animal or plant diversity, microbiologists could do little about it.

Caves are amongst the only environments protected from harsh surface conditions and may contain pristine evidence of microbial life (Cañaveras et al. 2001), which may serve as promising targets for survey of bioactive compounds. But in spite of an extensive phase of study of bioactivity in caves, not many antimicrobial compounds have been characterised in this unique atmosphere. In most instances, the chemical character along with the quantity of bioactive compounds generated by a particular bacterial strain is unknown. A quantum leap in culture-independent molecular phylogenetic approaches has created research on microbial groups in various ecosystems additionally achievable (Northup et al. 2011). Nevertheless, employing recent molecular methods only to learn equally identified and non-identified microbial populations in an ecosystem has its own restrictions. Quite a lot of investigations implicate that employing a blend of culture-independent and

culture-dependent techniques yields an additional rational picture of the native microbial diversity (Gurtner et al. 2000).

The intense ecosystems, considered as one of the apt resources of functional compounds, have been defined as habitats that experience steady or fluctuating exposure to one or more environmental factors, such as salinity, osmolarity, desiccation, UV radiation, barometric and hydrostatic pressures, pH, temperature, nutrient and available water constraint and trophic reliance on surface ecosystems (Seufferheld et al. 2008). As a result, these ecosystems necessitate from the microbes that thrive in them physiological transformations that are missing when cells exist in planktonic form (Barton 2006; Plath et al. 2007). A few of the studies have pivoted on sorting secondary metabolites generated by microbes that dwell in such ecosystems as possible basis of functional compounds: extremozymes, exopolysaccharides, biosurfactants, antitumour compounds, radiation-protective drugs, antibiotics, immunosuppressants and statins (Orme et al. 2001; Banerjee and Joshi 2013; Maria 2013). The investigations carried out on secondary metabolites from cave microbes the world over in the last 10 years are summarised in Table 1.1.

The subterranean (Latin subterraneus; from sub meaning "under" and terra meaning "earth") zone of the Earth is one of the main habitats that harbour a considerable proportion of microbial life. An effortless mode to come within reach of this ecosystem is to explore the hypogean environment, which is spread out to more than 20% of the Earth's subsurface. As caves are one of the major traits of karst topography, they may well throw some light into subsurface habitations (Engel and Northup 2008). It is generally known that caves can also accommodate a broad gamut of enthralling life types, ranging from biofilms [complex aggregation of microorganisms surrounded in a self-fabricated atmosphere and adhering to static or living surfaces (Banerjee et al. 2012)] to diverse kinds of creatures such as snails, worms, spiders, leeches, crickets, cockroaches, scorpions, fishes and bats (Maria 2013). In spite of being comparatively shielded from issues that, in surface ecosystems, have harmful effects on the survival of microbes, caves are considered as intense environments owing to the dearth of daylight and nutrient constraint (Maria 2013).

Several definitions have been applied to illustrate a cave. Generally, a cave, irrespective of its geological history and location, is defined as a natural hollow space in a rocky environment where at least a few portions of it are totally dark. The discipline of examination of caves and different karst characteristics is termed as speleology, a broad interdisciplinary science dealing with a thorough exploration of the creation of caves and their effects on their environments worldwide (Lee et al. 2012). The biology of a cave is, as a result, a mesmerising topic for investigating diverse facets of the hypogean environments and their relations with the surface environments. It can be mentioned at this juncture that it is to a certain extent a new forefront exclusively involving microbes, other living creatures and their relations with minerals that gives us a better grasp of the long-ago geomicrobiological chemical interactions (Baskar et al. 2011a).

Caves are inhabited by diverse groups of microorganisms such as bacteria, archaea, viruses and fungi (Bastian et al. 2010). The increased human interference due to tourism and consequent cave modifications (making of pavements and

Table 1.1 A glimpse of studies on secondary metabolites from caves during the last decade

Cave	Bioactivity	Microbe	References
Phatup Cave Forest Park and Phanangkhoi Cave, Thailand	Antibacterial, anticancer activity	*Spirillospora*, *Nonomuraea*	Nakaew et al. (2009)
Borra Caves, India	Anti-MRSA (methicillin-resistant *Staphylococcus aureus*), antitubercular activities	*Streptomyces* sp.	Radhakrishnan et al. (2014)
Krubera-Voronja Cave, Georgia	Antibacterial activity (the main antibacterial compounds produced by the strains were identified to be pyrrolopyrazines pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl), pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(phenylmethyl) and 1,2-benzenedicarboxylic acid, bis(2-methylpropyl) ester	Gram-positive bacteria belonging to the family *Bacillaceae* in phylum *Firmicutes* [unidentified]	Klusaite et al. (2016)
Azorean lava tubes of Terceira Island, Portugal	Antibacterial activity towards food-borne human pathogens (*Salmonella typhimurium*, *Escherichia coli*, *Staphylococcus aureus*, *Listeria monocytogenes*)	Bacterial isolates, majority of which belonged to Gram-positive (~70%) [unidentified]	Varela et al. (2009)
Karstic caves of Turkey	Antimicrobial activity against antibiotic-resistant clinical bacterial strains (methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant *Enterobacter faecium* (VRE), *Acinetobacter baumannii*)	*Streptomyces* sp.	Yücel and Yamaç (2010)
Sarawak limestone caves, Borneo	Quick constitutive producers of urease, potentially useful in inducing biocementation	Gram-positive bacterial isolates [unidentified]	Omoregie et al. (2016)
Caves of Brazil	Tannase producers	*Aspergillus japonicus*, *A. niger*, *A. tamarii*, *A. foetidus*, *A. tubingensis*, *A. ochraceus*, *Penicillium funiculosum*, *P. oxalicum*, *P. corylophilum*, *P. citrinum*, *Fusarium*, *Rhizopus*, *Epicoccum*, *Trichoderma*, *Cladosporium*	da Costa Souza et al. (2013)
Mandeepkhol Cave, India	Pectinolytic potency	*Brevundimonas diminuta*, *Bacillus thuringiensis*, *Bacillus cereus*	Karkun et al. (2012)

(continued)

Table 1.1 (continued)

Cave	Bioactivity	Microbe	References
Shenandoah National Park Cave, Virginia, USA	Biosynthesis of melanin	*Streptomyces* sp., *Arthrobacter* sp., *Lysinibacillus sphaericus*	Yousif et al. (2015)
Middle Siberian limestone caves	Biological control of phytopathogens (*Bipolaris*, *Fusarium*, *Alternaria*)	Psychrophilic and psychrotolerant bacteria and fungi [unidentified]	Khizhnyak et al. 2003
Middle Siberian limestone caves	Amylase producers	Cold-adapted amylolytic fungi of the genus *Geomyces*	Khizhnyak et al. 2003
Frasassi cave system, Italy	Secretion of exopolymeric substances (EPS), sulphuric acid generation, removal of arsenic	Acidophilic S-oxidising bacterial strains of *Acidithiobacillus thiooxidans*	Beolchini et al. (2017)
Gumki Cave, India	Producers of lipase, amylase, protease, cellulase	Bacterial genera belonging to *Bacillus*, *Paenibacillus*, *Staphylococcus*, *Streptococcus*, *Salimicrobium*, *Lysinibacillus*, *Aeromonas*, *Proteus*, *Clostridium*	Rautela et al. (2017)
Khangkhui Cave, India	Antibiotic activity	Actinobacterial strains	Ningthoujam et al. (2009)

installation of lighting systems), however, can alter their microclimatic conditions and natural microbial dynamics besides introducing exotic microbes. These changes over time can cause extensive damages to cave features such as Palaeolithic paintings and finger flutings. The ever-increasing number of tourists can also lead to increased health risks to cave visitors and workers via increased microbial load and exposure to opportunistic cave pathogens. Caves represent a distinctive pristine niche that may harbour novel microbial species that might produce biomolecules such as enzymes and antibiotics having applications in future biotechnological endeavours. The stressed cave microclimatic conditions may enhance the production of different biomolecules (Kay et al. 2013). The bacterial hydrolytic enzymes have drawn the curiosity of microbiologists for an array of future industrial uses (Adrio and Demain 2014). Enzymes like chitinases, gelatinases, xylanases, cellulases, inulinases, pectinases, phytases, xylanases and DNases find relevances in bioethanol production, waste upcycling, household care, biotechnology, pharma, cosmetics, paper, textile and pulp industries, in addition to human and animal nourishment (Riquelme et al. 2017).

In this chapter, we have tried to describe the cave environments and provide sufficient information for considering them as extreme environments for life. Recent updates on geomicrobiological studies have been highlighted, and their biotechnological prospectivity has been revealed. The opportunities in employing cave microbes to fabricate innovative medicines are enlisted along with future perspectives on this subject.

1.2 The Hypogean Environment

Cave environments have been utilised by individuals and creatures for hundreds of years. Archaeological studies have documented caves used yet in the Palaeolithic era. Human beings have used caves for an array of activities, for example, for refuge, as origin of easily available minerals, as spots for exhibiting paintings and as burial spots, to name a few. During recent times, however, humans have used caves mainly for recreation and scientific exploration (Ghosh et al. 2017). There are evidences that prove the inhabitation of this pristine stressed niche by a variety of forms of existence (Taylor 1999). In subterranean habitations like caves, the means of metabolism are strikingly dissimilar to enable the organisms to cope up with the varied challenges that include partial accessibility of organic material, inconsistent intensity of luminosity and moisture, restricted connectivity to the outside, fluctuations in temperatures, contact with humans or animals, air surge and state of pressure, along with the mineral environment of the caves (Ghosh et al. 2017; Engel 2011; Barton 2015).

Karst environments, in which the majority of the caves exist, encompass roughly 15–20% of Earth (Ford and Williams 2007). A meagre 10% of all caves around the world have been investigated (Engel 2011). Approximately 350 minerals from caves have been documented, few of which are latest addition to science (Onac and Forti 2011). Each cave is distinctive in its biological, chemical and physical features. Caves have been categorised based on speleogenesis, i.e. how a cave is geologically shaped and constructed. The criteria used for cave classification include (a) kinds of minerals and adjoining bedrock (e.g. limestone or lava tube), (b) geometric configuration and morphology (e.g. horizontal or vertical), (c) time when formed in relation to the rock in which they originated (e.g. primary, secondary or tertiary) and (d) the means by which they were created (e.g. solutional or non-solutional) (Engel 2011). Depending mainly on the intensity of light that penetrates the cave, it can be partitioned into four key regions: (1) entrance (where the surface and underground environments congregate), (2) twilight (where light slowly diminishes to zero and no plants can thrive further than this region), (3) transition (where there is no light, but surface environmental changes such as temperature and moisture can still be noted) and (4) deep (where it is totally dark, with soaring humidity and steady temperature) (Ghosh et al. 2017).

The mysterious microbial kingdoms inhabiting caves depend on other bases of energy that they derive from the adjacent atmosphere, minerals and rocks due to the absence of light and consequent universal basis of energy supplied in the course of

photosynthesis (Banerjee and Joshi 2013). The deep cave environments, therefore, are extremely oligotrophic in nature (Lee et al. 2012).

Various aspects manipulate the total heterogeneity within and amongst cave environments accordingly, making each cave habitat unique in it (Onac and Forti 2011). In these virgin environments, autotrophism can only be encountered in the entrance, where light is still the primary resource of energy input. The autotrophic way of life steadily becomes less common in the deeper zones owing to diminishing light intensity. Thus, oligotrophs and heterotrophs, including predators, colonise the deeper cave habitats guided by the source and amount of available energy (Ghosh et al. 2017). Any environment that contains a mere 2 mg/L of total organic carbon (TOC) is considered as oligotrophic (Barton 2015). Caves commonly receive organic carbon as dissolved organic carbon (DOC) that is made available in the caves as a consequence of allochthonous inputs from the exterior soil-derived environments (Barton 2015).

Although cave ecosystems are extremely nutrient-restricted, different microbes that exhibit erratic enzymatic and antimicrobial properties are still able to thrive (Cheeptham 2013). The cultivation of these microorganisms has posed a real challenge for microbiologists. At the outset, scientists devised nutrient-rich media and incubated them at 37 °C, resulting into reduced revival (Lavoie 2015), which is attributed to their adaptability to live in nutrient-starved ecosystems and be obliged to scavenge to get food. The great quantity of nutrients in standard media guides to osmotic pressure in those microbial cells from caves, resulting in microbial death (Ghosh et al. 2017). More recent accepted methodologies involve the employment of low-nutrient media and low temperature (15 °C) for cultivation of cave microbes (Lavoie 2015).

1.3 Microbial Diversity in Caves

Ecological relations markedly influence the improvement and safeguarding of the dynamics involved in cave environments (Lee et al. 2012). Based on the environmental circumstances, cave organisms can either be motile or sessile. They can directly or indirectly remain involved symbiotically (parasitic and mutualistic) with other organisms (Macalady et al. 2007). Further, based on the setting and state in the cave, organisms may be either non-residents or permanent residents (Romero 2009). Non-resident organisms, termed as accidentals, move into a cave sporadically through wind, water, air, sediment or animals. Based on their acts and duration of visit in the cave, they may have a significant effect on the native inhabitants (Culver and Sket 2000). The means for the source of these native inhabitants still needs to be discussed; the closest possible justification is that they firstly came down from the exterior and ventured the subterranean surface either inadvertently or were strained below by any disastrous incidents. As soon as they are down, they become accustomed to the prevailing environmental conditions (Lee et al. 2012). Although exhaustive studies on microbial diversity in caves have been documented internationally, such efforts have not been made at the national level. Table 1.2 presents geomicrobiological studies carried out in Indian caves.

Table 1.2 An overview on microbial diversity studies in the cave environments of India

Cave	Sampled from	Microorganisms involved	References
Caves from Jaintia and East Khasi Hills, Meghalaya	Spring water, rock samples	Gram-positive, Gram-negative bacteria	Sheikh et al. (2013)
Sahastradhara Caves, Siwalik Himalaya, Dehradun, Uttarakhand	Stalactites, moonmilk	*Bacillus pumilus*, *B. cereus*, *B. anthracis*, *B. lentus*, *B. sphaericus*, *B. circulans*, *B. thuringiensis*, sulphate-reducing bacteria, archaea, actinomycetes; microbial forms similar to *Cyanobacteria* and *Spirulina*	Baskar et al. (2005, 2006, 2014)
Mawmluh Cave, Meghalaya	Moonmilk and moonmilk pool waters	*Bacillus cereus*, *B. licheniformis*, *B. pumilus*, *Micrococcus luteus*, actinomycetes	Baskar et al. (2011a, b)
Mawsmai and Phyllut Caves, Sohra, East Khasi Hills, Meghalaya	Speleothems, cave wall deposits	*Bacillus cereus*, *B. mycoides*, *B. licheniformis*, *Micrococcus luteus*, actinomycetes	Baskar et al. (2009)
Borra Caves, Visakhapatnam, Andhra Pradesh	Speleothems, microbial mats of the spring waters	*Leptothrix*-like and *Gallionella*-like organisms, microbes belonging to the phyla *Proteobacteria*, *Chloroflexi*, *Bacteroidetes*, *Planctomycetes*, *Actinobacteria*, *Acidobacteria*, *Nitrospira*, *Firmicutes*	Baskar et al. (2007, 2008, 2012)
Caves of southern India	Cave soil	Fungi belonging to the genera *Aspergillus*, *Penicillium*, *Absidia*, *Rhizopus*, *Mucor*, *Chaetomium*, *Sepedonium*	KoilRaj et al. (1999)
Pannian, Samanar, Ushman and KKB Caves at Madurai, Tamil Nadu	Cave soil	*Bacillus* sp., *Clostridium* sp., *Serratia* sp., *Pseudomonas* sp. *Nitrosomonas* sp., *Nitrobacter winogradskyi*, *Nitrococcus mobilis*; mesophilous fungi (belonging to deuteromycetes, zygomycetes and ascomycetes) and thermophilous fungi	KoilRaj et al. (2012)
Rani Cave, Chhattisgarh, India	Speleothems	*Bacillus subtilis*, *Cupriavidus* sp.	Chalia et al. (2017)
Gumki Cave, Nandakini River Valley, Chamoli District Uttarakhand	Stalactite, white mat (microbial mat), brown mat (iron crust), water and wall scrapping	Bacterial genera belonging to *Bacillus*, *Paenibacillus*, *Staphylococcus*, *Streptococcus*, *Salimicrobium*, *Lysinibacillus*, *Aeromonas*, *Proteus*, *Clostridium*	Rautela et al. (2017)

(continued)

Table 1.2 (continued)

Cave	Sampled from	Microorganisms involved	References
Kotumsar Cave, Kanger Valley National Park, Bastar District, Chhattisgarh	Speleothems	*Rhodococcus* sp.	Baskar et al. (2018)
Khangkhui Cave, Ukhrul District, Manipur	Cave soil	Actinomycete strains	Ningthoujam et al. (2009)
Mandeepkhol Cave, Salewara Mountain Range, Chhattisgarh, India	Guano, leaf litter and wood deposits	Fungal species belonging to *Zygomycotina*, *Ascomycotina*, *Deuteromycotin*a	Karkun et al. (2012)
Kotumsar Cave, Kanger Valley National Park, Bastar District, Chhattisgarh	Various depth-dependent microhabitats of cave	*Streptomyces prasinosporus*, *Micrococcus luteus*, *M. radiodurans*, *M. agilis*, *Stomatococcus mucilaginosus*, *Staphylococcus sciuri*, *S. varians*, *S. simulans*, *S. epidermidis*, *Deinococcus radiodurans*, *Streptococcus*	Rajput et al. (2012a, b), Rajput and Biswas (2012) and Rajput et al. (2014)
Borra Caves, Visakhapatnam, Andhra Pradesh	Cave soil	*Streptomyces* sp.	Radhakrishnan et al. (2014)
Rongai Dobhakol Cave, Garo Hills, Meghalaya	Various cave habitats	Aerobic rod-shaped Gram-positive bacteria	Baskar and Baskar (2014)
Krem Syndai, Jaintia Hills, Meghalaya	Speleothems	*Bacillus* sp., *Actinomycetes* sp., *Streptomyces* sp., *Pseudomonas* sp., *Micrococcus* sp., *Staphylococcus* sp., *Xanthobacter* sp., *Arthrobacter* sp.	Baskar et al. (2016)
Soitan, Mawpun and Lawbah Caves, Khasi Hills, Meghalaya	Speleothems	*Bacillus simplex*, *B. gaemokensis*, *B. subtilis*, *B. thuringiensis*, *B. albus*, *B. cereus*, *B. anthracis*, *B. weihenstephanensis*, *B. wiedmannii*, *Rummeliibacillus stabekisii*, *R. pycnus*, *Staphylococcus epidermidis*, *Kurthia zopfii*, *Brevibacterium frigoritolerans*	Mudgil et al. (2018)

(continued)

Table 1.2 (continued)

Cave	Sampled from	Microorganisms involved	References
Mawsmai Cave, Sohra, East Khasi Hills, Meghalaya	Stalactite, stalagmite, column	*Pseudomonas gessardii*, *P. vranovensis*, *P. chlororaphis* subsp. *aurantiaca*, *P. taiwanensis*, *P. mosselii*, *Bacillus vallismortis*, *B. subtilis* subsp. *inaquosorum*, *Lysinibacillus macroides*, *Brevibacillus agri*, *Ensifer adhaerens*, *Staphylococcus saprophyticus* subsp. *bovis*, *Acinetobacter johnsonii* and *Kocuria rosea*	Banerjee and Joshi (2014, 2016)
Mawmluh Cave, Sohra, East Khasi Hills, Meghalaya	Flowstone, cave wall deposit	*Kurthia gibsonii*, *Bacillus halodurans*, *Aeromonas hydrophila* subsp. *hydrophila*, *Flavobacterium chungangense*, *Staphylococcus equorum* subsp. *equorum* and *Pseudomonas monteilii*	Banerjee and Joshi (2014, 2016)
Mawjymbuin Cave, Mawsynram, East Khasi Hills, Meghalaya	Stalactite, cave wall deposit	*Bacillus amyloliquefaciens* subsp. *amyloliquefaciens*, *B. cereus*, *Iodobacter fluviatilis*, *Lysinibacillus parviboronicapiens*, *Pseudomonas alcaligenes* and *Brevibacillus agri*	Banerjee and Joshi (2013, 2014, 2016)
Dam Cave, Mawsynram, East Khasi Hills, Meghalaya	Cave wall deposit, flowstone	*Sphingobacterium kitahiroshimense*, *S. faecium*, *Brevibacillus agri*, *Achromobacter xylosoxidans* and *Bacillus* sp.	Banerjee and Joshi (2013, 2014, 2016)
Labit Cave, Shnong Rim Area, East Khasi Hills, Meghalaya	Stalactite, stalagmite	*Bacillus thuringiensis*, *B. circulans*, *B. isronensis*, *B. cereus*, *Paenibacillus massiliensis* and *Brevibacterium frigoritolerans*	Banerjee and Joshi (2014, 2015, 2016)

1.3.1 Viral Diversity

Viruses are largely the most rich type of biological entity on Earth, being encountered wherever there is life, and have almost certainly existed since the first cells developed (Kyle et al.2008). They have a strong impact on virtually all evolutionary and ecological processes. They can infect all types of organisms from prokaryotes to plants and animals (Abedon 2008). In spite of their versatility, there have been meagre studies on their impact on cave ecology (Ghosh et al. 2017).

Till now, the occurrence of viruses in these subterranean ecosystems has only been exhibited in a few incidents. In 1998–1999, 154 miners employed in a gold mine in the Democratic Republic of Congo reportedly caught the Marburg haemorrhagic fever. The causative virus for this disease was isolated in 2007 from African

bats, though the reason for this infection was unknown owing to unclarity in the source of initial infection (Kuzmin et al. 2010). But, no investigation has till now been carried out on viral diversity using environmental samples from caves. These viruses could also be considered as beneficial driving forces due to their prospective lytic characteristics (Ghosh et al. 2017). Thus, it can be assumed that the cave environment may also contain a massive load of viruses that needs to be assessed.

1.3.2 Bacterial and Archaeal Diversity

The prokaryotes, which encompass the domains *Bacteria* and *Archaea*, are mainly a rich cluster of organisms on our planet. It can steadily be taken into account that these microbial clusters have enacted a significant task in the primordial advancement of our planet (Lee et al. 2012). Till now, majority of investigations on cave microbial ecology have highlighted on bacterial diversity from mineral deposits found on the walls and ceilings of caves and on speleothem surfaces (Ghosh et al. 2017). Recent works by the investigators have demonstrated the capabilities of the bacterial strains from the caves of Meghalaya, India (which are some of the longest caves in the subcontinent that have so far received negligible attention from geomicrobiologists), to precipitate calcite providing substantiation for biotic practices engaged in formation of speleothems (Banerjee and Joshi 2014). Thirty-two different culturable bacterial species belonging to 16 different genera were characterised from 5 caves of the region. Based on molecular identification, the isolates were related to nearest taxa, with the majority belonging to *Bacillus* and *Pseudomonas* (Banerjee and Joshi 2015, 2016).

The amount of bacterial and archaeal 16S rRNA gene sequences recovered by model clone libraries and pyrosequencing of a range of samples collected from different cave environments globally constitutes a meagre portion of all the estimated 16S rRNA gene sequences (Engel 2010). According to Lee et al. (2012), roughly half of the bacterial phyla, and not as much than half of the archaeal phyla, inhabiting caves globally are characterisable to a definite level. The remaining 16S rRNA gene sequences so far recovered from cave environments correspond to new to date unculturable species with unidentified roles (Lee et al. 2012). A cultivation-based approach investigated by Velikonja et al. (2014) inspected the microscopic microbial colonies having distinct colouration (cave silver) from a karstic cave in Slovenia using 16S rRNA gene profiling. They recovered 80 isolates, majority of which were identified as *Streptomyces* (25%), *Micrococcus* (16%) and *Rhodococcus* (10%). Other bacteria identified were *Pseudomona*s (9%), *Agrobacterium* (8%), *Lysobacter* (6%) and *Paenibacillus* (5%), while *Microbacterium*, *Agrococcus*, *Arthrobacter*, *Bacillus*, *Kocuria*, *Oerskovia*, *Sphingomonas*, *Aerococcus* and *Bosea* were present in only very low frequency. In another study, 474 16S rRNA gene clones were recovered from the metagenomic DNA extracted from yellow microbial communities collected from three limestone caves located in Spain, the Czech Republic and Slovenia. Statistical breakdown of the figures showed the existence of bacteria

fitting to the family *Pseudonocardiaceae* (30–50%), *Chromatiaceae* (6–25%) and *Xanthomonadaceae* (0.5–2.0%) (Porca et al. 2012).

Next-generation sequencing (NGS) has also been applied to study the cave microbial diversity. For example, a 454-pyrotag examination of the V6 region of the 16S rRNA genes was analysed to scientifically assess the bacterial diversity of ten speleothem samples from Kartchner Caverns, a limestone cave (Ortiz et al. 2013). Comparative analysis of speleothems showed three prominent bacterial taxonomic profiles governed by either *Actinobacteria*, *Proteobacteria* or *Acidobacteria*. The sampling points that showed microbial communities with less diversity were governed by *Actinobacteria*, whereas in the more varied communities, *Proteobacteria* were prevalent. Moreover, 16S clone library depiction of a speleothem model of the Kartchner Caves showed *Actinobacteria* to be the prevalent bacterial population, of which 99% comprised of chemoautotrophs and oligotrophs. Simultaneously, pyrosequencing of the small subunit rRNA genes and examination of bacterial abundance in Jinjia Cave, a limestone cave situated on the Loess Plateau of China, revealed dominance by cave-specific bacterial ancestries surrounded by *γ-Proteobacteria* or *Actinobacteria* (Wu et al. 2015). These two reports (Kartchner and Jinjia Caves) thereby exposed almost similar microbial communities, with only minor differences, in spite of being in diverse geographical sites (eastern North America and eastern Asia, respectively). In a separate study, Riquelme et al. (2017) have created the most up-to-date portrayal of the diversity of actinomycetes in volcanic caves from two islands in the Azores, Portugal, as well as Hawaii and New Mexico, USA, by means of scanning electron microscopy (SEM) and Sanger sequencing 454 pyrosequencing. Even though this investigation revealed the incidence of a diversity of microorganisms belonging to the order *Actinomycetales*, additional orders such as *Euzebyales*, *Rubrobacterales*, *Solirubrobacterales*, *Coriobacteriales* and *Gaiellales* were also encountered.

1.3.3 Fungal Diversity

Fungi are not dispersed consistently all through the length and breadth of caves. Bulk of the fungal genera accounted from cave ecosystems are saprotrophs; the majority of them live in the karst surface, colonise decaying organic matter and cover the proliferation of oligotrophic cave fungi (Ghosh et al. 2017). Many fungi that have been cultured from the cave environment are not native to caves (Novakova 2009). They are transported to the cave environment by water, air currents or animals. They can as well be instrumental to the development of speleothems, such as secondary calcium carbonate deposits (Bindschedler et al. 2012). Mycorrhizal fungi have also been observed in plant roots that infiltrate shallow caves (Jasinska et al. 1996). They, however, could not be identified.

Most of the workers have identified *Ascomycota* as the most abundant fungal phylum inhabiting caves. Genera like *Penicillium*, *Aspergillus*, *Botrytis*, *Cladosporium* and *Fusarium* are the most common ones (Man et al. 2015; Pusz et al. 2015) colonising the caves. A lethal fungal disease, white nose syndrome (WNS), reported in cave-dwelling North American bats in 2006, has enthused

microbiologists to study cave fungi (Lindner et al. 2011). It has been reported that WNS is caused by a recently described fungus *Geomyces destructans*; however, till date it is not clear if *G. destructans* is native to North America or is a newly introduced exotic species (Vanderwolf et al. 2013). *G. destructans* has also been characterised from caves in Europe from where no death of bats has till now been accounted (Northup and Lavoie 2015).

Mulec et al. (2012) reported that caves generally lack the rich fungal diversity commonly reported from other natural ecosystems (Mulec et al. 2012). Vanderwolf et al. (2013) reviewed the fungal diversity of caves worldwide and concluded that the fungal taxa reported from caves constituted of 69.1% *Ascomycota*, 20% *Basidiomycota*, 6.6% *Zygomycota*, 2.6% *Mycetozoa*, 1% *Oomycota* and 0.8% others (*Amoebozoa*, *Chytridiomycota*, *Microsporidiomycota* and *Percolozoa*). The investigators documented that the genera most frequently reported from studies on cave mycology, aside from *Histoplasma* and *Geomyces*, are *Aspergillus* (38 out of 60 locations), *Penicillium* (36), *Mucor* (29), *Fusarium* (27), *Trichoderma* (25), *Cladosporium* (23), *Alternaria* (21), *Paecilomyces* (21), *Acremonium* (19), *Chrysosporium* (19), *Laboulbenia* (18), *Rhizopus* (18), *Mortierella* (17), *Chaetomium* (16), *Rhachomyces* (16), *Trichophyton* (15), *Humicola* (14), *Isaria* (14), *Absidia* (13), *Beauveria* (13), *Phoma* (13), *Verticillium* (13), *Aureobasidium* (12), *Gliocladium* (12), *Coprinus* (11), *Cunninghamella* (11), *Epicoccum* (11), *Geotrichum* (11), *Microsporum* (11), *Botrytis* (10), *Candida* (10), *Mycena* (10), *Scopulariopsis* (10) and *Stachybotrys* (10).

Several studies have been carried out to observe the impact of human visitors to cave fungal diversity. Vaughan-Martini et al. (2000) observed that an Italian cave with record human footfall (~400,000 visitors/year) had more yeast strains than two other caves with low human visitation (a few speleologists/year). Shapiro and Pringle (2010), however, have observed that caves with record human footfall had lesser fungal diversity in contrast to the caves with moderate footfall. This observation is in contrast with the observations of Vaughan-Martini et al. (2000). It is also possible that microclimatic changes due to human visitation and lighting systems could impact fungi in caves (Vanderwolf et al. 2013).

1.4 Bioprospection of Cave Microbes

Bioprospecting, hallmarked as a source of finance for biodiversity conservation, is the process of discovery and commercialisation of new products based on biological resources (Galvez et al. 1993). Despite indigenous knowledge being intuitively helpful, bioprospecting has only recently begun to incorporate such knowledge in focusing screening efforts for bioactive compounds (Saslis-Lagoudakis et al. 2012). From the deep, dark depths of caves to the red-hot centre of volcanoes, researchers are bioprospecting extreme environments for antimicrobials. Due to the immense prospective for finding novel drug (Knight et al. 2003), studies on bioactivity of metabolites produced by cave-dwelling microbes began 30 years ago and are still in progress; consequently, caves are studied all over the globe, i.e. Europe, Asia and North America. More or less 45% of all recognised microbial bioactive compounds are

generated by members of phylum *Actinobacteria*, and almost 80% of these bioactive compounds are manufactured by the genus *Streptomyces* (Ghosh et al. 2017). Discovery of novel antibiotics is vital owing to the appearance of fresh races of multi-drug-resistant pathogens. Consequently, substantial numbers of investigations on bioactivity of cave microbes have been carried out on this group of prokaryotes (Maciejewska et al. 2015). Since the last 4 years, microbiologists have started paying attention to other bacterial phyla such as *Bacteroidetes*, *Firmicutes*, *Proteobacteria* and *Cyanobacteria* characterised from caves for bioactive metabolites (Tomova et al. 2013, Lamprinou et al. 2015) besides the phylum *Actinobacteria*.

Molecular techniques have also been employed for the assessment of the biosynthetic potential of cave microorganisms. Hodges et al. (2012) reported that most of bioactive molecules are the results of either polyketide synthases (PKS) or non-ribosomal peptide synthetases (NRPS). PKS and NRPS gene sequences were expected to correspond to angucycline (an antitumour antibiotic), the antibiotics naphthoquinone and β-lactam as well as cyclohexadepsipeptide and siderophores. The investigators successfully applied these gene clusters to aim primers in bacterial isolates from Mystery Cave and Norman's Cave (Bahamas) and identified these clusters in strains belonging to the genera *Streptomyces*, *Nocardiopsis*, *Solwaraspora* and *Micromonospora*.

Some recent scientific reports related to the function of cave microbes in the production of bioactive metabolites that can be used for human well-being are being described below.

1.4.1 Moonmilk Microbiome as Source of Novel Compounds

The moonmilk is comparatively an unusual speleothem in cave ecosystems. It develops as a solid calcite paste, and a minor amount of aragonite, hydromagnesite, monohydrocalcite, nitrates and sulphates, in passageways that receive significant airflow (Borsato et al. 2000). It is white to grey in colour. Contrary to stalactites and stalagmites, the moonmilk displays a spongy consistency of microcrystalline collections. The exact process of moonmilk formation remains unclear. However, high abundance of filamentous bacterial cells and members of the *Actinobacteria* has led researchers to suggest a biogenic origin of moonmilk (Portillo and Gonzalez, 2011). Its therapeutic activities may possibly be connected with the existence of abundant filamentous *Actinobacteria*, predominantly *Streptomyces*. The characterisation of prospective new compounds with broad-spectrum properties from this genus maintains the proposal of moonmilk being an excellent object for viewing bioactivity (Frère and Rigali 2016).

Notwithstanding its comparative paucity in caves, moonmilk has extensive logical curiosity due to its historical use as a medical treatment. It is recognised that moonmilk was applied to mend bone fracture and chancre, apart from being employed as a medicine for diarrhoea and dysentery. In veterinary medication, moonmilk was made use of as a drug for cows with decreased lactation (Reinbacher 1994). Nevertheless, the clear-cut consequences of the medicine in addition to the

geochemical characteristics of moonmilk remain unidentified (Mazina and Semikolennykh 2010).

Axenov-Gibanov et al. (2016) characterised ten actinobacterial strains from moonmilk and from a subterranean lagoon situated in one of the largest conglomeratic karstic caves in the planet. The study demonstrated that isolates belonging to the genera *Streptomyces* unveiled antibacterial and fungicidal properties. A few strains were also capable to hinder the multiplication of pathogenic *Candida albicans*. According to the investigators, these strains are of specific interest and require detailed investigations, including isolation and characterisation of pure active compounds and investigation of their biosynthetic machinery (Axenov-Gibanov et al. 2016).

A screening approach was undertaken by Maciejewska et al. (2016) to characterise culturable *Actinobacteria* from moonmilk of the Grotte des Collemboles in Belgium. The investigators worked on the taxonomic profile of the isolates and evaluated their possibility in biosynthesis of antimicrobials. Phylogenetic study showed that all 78 strains belonged to the genus *Streptomyces* and are grouped into 31 discrete phylotypes exhibiting different pigmentation blueprints and morphological characteristics. Phylotype representatives were checked by the investigators for antibacterial and antifungal properties, and their genomes were used for identification of secondary metabolite biosynthetic genes coding for non-ribosomal peptide synthetases (NRPS) and polyketide synthases (PKS). The investigators noted that moonmilk *Streptomyces* exhibited powerful inhibitory properties towards a vast array of reference microbes. 94, 71 and 94% of *Streptomyces* strains weakened the multiplication of Gram-positive and Gram-negative bacteria and fungi, correspondingly. It was found out that 90% of the strains brought about maximum reduction in growth towards the multi-drug-resistant *Rasamsonia argillacea*, a causative agent of invasive mycosis in cystic fibrosis and chronic granulomatous diseases. Overall, their work supported the common belief that moonmilk might effectively treat various infectious diseases and, thus, may indeed constitute a promising reservoir of potentially novel active natural compounds (Maciejewska et al. 2016).

1.4.2 Breakdown of Organic Wastes and Reprocessing of Nutrients

Caves accommodate a range of microbes which participate in a vital function in disintegrating organic rubble of cave animals and reprocess the food chain in the cave ecosystem (Chelius et al. 2009; KoilRaj et al. 2012). The nutrient cycling in cave totally depends on the catabolic and anabolic practices of bacteria and fungi (Alfreider et al. 2003). The heterotrophic microbes derive carbon from preformed organic compounds that are broken down enzymatically, while the chemoheterotrophs derive energy through the oxidation of organic compounds via respiration, and they are critical to biogeochemical cycling, waste treatment and bioremediation (KoilRaj et al. 2012).

Breakdown of organic wastes and reprocessing of nutrients also get enhanced by the activity of both bacterial and fungal species accessible at the entrance, twilight and dark regions of caves. Based on this hypothesis, four different caves, namely,

Pannian, Samanar, Ushman and KKB, located at Madurai were examined by KoilRaj et al. (2012). On the basis of their results, KoilRaj et al. (2012) concluded that the roles played by chemoheterotrophic and chemoautotrophic bacteria were essential for the maintenance of cave environment. The majority of chemoheterotrophs characterised by them were species of ammonifiers such as *Bacillus* sp., *Clostridium* sp., *Serratia* sp. and *Pseudomonas* sp., while nitrite bacteria similar to *Nitrobacter winogradskyi* and *Nitrococcus mobilis* gained energy from the oxidation of ammonia and nitrite, respectively. The results of the FT-IR spectrum carried out by the investigators showed that stretching of mineralised compounds occurred because of microbial role with the help of enzymes which in due course disintegrate the intricate organic matter into simpler structures.

1.4.3 Removal of Arsenic by Sulphur-Oxidising Cave Bacteria

Metals and metalloids (semi-metals) are of concern due to their non-biodegradability, persistence and toxicity (Brierley and Brierley 2013). Unlike organic pollutants, they cannot be degraded; however, they are not permanently adsorbed to the sediment and can spread into the environment as a result of redox changes and/or resuspension during disposal actions (Pastorelli et al. 2012). Bioleaching (i.e. a biological technique in which acidophilic microorganisms catalyse metal solubilisation from ores) has been considered as a promising and environment-friendly strategy for the removal of metals from contaminated dredged sediments (Akcil et al. 2015).

Beolchini et al. (2017) isolated acidophilic S-oxidising bacterial strains from snottites, extremely acidic (pH 0–1) and viscous biofilms (Jones et al. 2012), from sulphur-rich deep Frasassi cave system characterised by low temperature (Italy). They studied the ability of the isolates to mobilise (semi-)metals from contaminated sediments. The snottites of Frasassi cave system were dominated by *Acidithiobacillus thiooxidans* (about 70% of the total cells) and were responsible for CO_2 fixation, secretion of exopolymeric substances (EPS) and sulphuric acid generation. The experiments of Beolchini et al. (2017) revealed that S-oxidising bacterial strains of *A. thiooxidans* had high potential to remove As from contaminated marine sediments. This was never reported before. These strains could be exploited for developing selective bioremediation procedures for removal of As from contaminated sediments and can also be applied in multistep biotreatment processes.

1.4.4 Importance of Cave Microbes in Production of Industrially Important Enzymes and Pigments

1.4.4.1 Application of Cold-Adapted Bacterial and Fungal Isolates

The cold-adapted bacterial and fungal isolates have attracted immense curiosity for low-temperature biotechnology, for biological plant defence in boreal temperature areas and in Bioregenerative Life Support Systems (BLSS) based on higher plants. The Middle Siberian limestone caves are usual pool of cold-adapted bacteria and

fungi (Khizhnyak et al. 2003). Cold-adapted bacterial and fungal strains were isolated from Karaulnaya-2 that are applied for biological remediation of phytopathogens like *Bipolaris*, *Fusarium* and *Alternaria.*

Bioregenerative Life Support Systems (BLSS) are devised for long-term existence of human sustainability by reusing biogenic constituents since it occurs in the normal environments. A major dilemma of BLSS on higher plants is their defence against infections. The selectivity of BLSS does not consent to spraying chemical pesticides for remediation of phytopathogens since these are noxious for humans. The biological means which are employed for disease management of plants as a substitute to chemical formulations are potentially dangerous for individuals if they are sprayed in closed surroundings. The cold-acclimatised microbes do not multiply at human body temperature and, therefore, crack the difficulty of plants defence in BLSS.

Khizhnyak et al. (2003) isolated nine strains of cold-acclimatised bacteria from the Karaulnaya-2 Cave. These isolates could not grow at human body temperature and suppressed conidia germination of *Fusarium oxysporum*. Fifteen and twelve strains, respectively, suppressed conidia germination of *Bipolaris sorokiniana* and *Alternaria* sp. Failure of these strains to multiply at optimum body temperature makes them suitable for biological remediation of phytopathogenic fungi in BLSS.

Amylases produced by psychrophilic and psychrotolerant microbial strains remain active at very low temperature (Kuddus et al. 2011). Amylases are one of the main enzymes used in industries and, therefore, account for approximately 25% of the world enzyme market (De Souza 2010). Up to now such amylases are produced by strains isolated from Antarctica, Arctic, high-mountain and deep-water ecosystems. Cold limestone caves, however, can also serve as a natural source of the psychrophilic and psychrotolerant bacteria and fungi. Two strains of cold-adapted amylolytic fungi of the genus *Geomyces* were isolated from low-temperature karst cave Karaulnaya-2 (Khizhnyak et al. 2003).

1.4.4.2 Biosynthesis of Melanin

Moving charges due to electric current create electromagnetic waves, which generate electromagnetic fields (EMFs). The low-frequency EMFs are unlikely to cause adverse effects on growth, gas production and cellular division of living organisms (Yousif et al 2015). Yet, data from research on epidemiological studies indicated ever-increasing frequency of cancer in living organisms exposed to low-frequency EMFs (Moulder and Foster 1995; Gurney and van Wijngaarden 1999). On the beneficial aspect, bio-stimulation through EMF in microorganisms may perhaps direct to benefits in the bioenergy sector and manufacture items of industrial and therapeutic importance (Yousif et al 2015). Yousif et al. (2015) hypothesised that a diverse variety of microorganisms, i.e. bacteria, will respond to EMF and bio-stimulate microbial metabolic products of commercial significance. 16S rRNA sequencing was carried out by the investigators to characterise three EMF-resistant isolates from soil samples collected from Shenandoah National Park Cave, Virginia, USA: *Streptomyces* sp., *Arthrobacter* sp. and *Lysinibacillus sphaericus*. They also investigated the bio-economics of melanin (a class of pigments which are widespread in

the animal and vegetable kingdoms) biosynthesis, by submerged fermentation and EMF contact, which revealed that waste shredded paper followed by starch was amongst appropriate substrates for biosynthesis of melanin under non-EMF situation. Melanins have very diverse roles and functions in various organisms. In case of microorganisms (such as bacteria and fungi), they protect against stresses that entail cell injury such as UV emission from the sun and reactive oxygen species. It also defends against injury from elevated temperatures, chemical stresses and biochemical hazards (Hamilton and Gomez 2002). As a result, in several pathogenic microbes (*Cryptococcus neoformans*), melanins seem to engage in prominent functions in virulence and pathogenicity by defending the microbes against immune reactions of the host.

1.4.4.3 Degradation of Pectin

Pectin is an abundant structural component of plant cell walls and functions as a matrix holding cellulose and hemicellulose fibres. Pectinases are cluster of enzymes that hydrolyse pectin and depolymerise it by hydrolysis and transelimination in addition to de-esterification reactions, which hydrolyse the ester bonds between carboxyl and methyl groups of pectin (Ceci and Lozano 1998). Pectinases have extensive relevance in fruit juice production and wine manufacturing and account for 10% of worldwide industrial enzymes manufactured and their promotion is ever increasing (Stutzenberger 1992). They are used in the paper and pulp industry besides cellulases (Reid and Ricard 2000).

A key role of bacteria is based on their ability to break down the key plant constituents mainly cellulose, hemicelluloses, pectin and lignin. Bacteria are potent decomposers and nutrient recyclers of forest litter. Without decomposing bacteria, we would soon be buried in debris. Moreover, plants are the major locking living entities on Earth which lock nearly 80–90% carbon of the atmosphere. These safe and sound intricate organic materials must be broken down up to simple monomers in order that they can be reprocessed by other organisms, thus helping in cycling of organic nutrients.

Keeping in tune with the above principle, a total of 32 bacterial species capable of digesting pectin were isolated by Karkun et al. (2012) from Mandeepkhol Cave located in a deep forest in Chhattisgarh, India. The assessment was done based on colonial growth and colonial dry weight. The results showed that *Brevundimonas diminuta*, *Bacillus thuringiensis* and *Bacillus cereus* possessed greater pectinolytic potency (Karkun et al. 2012).

1.4.4.4 Production of Tannase

Tannase (EC 3.1.1.20) is an enzyme that hydrolyses ester and depside links in gallotannins to release gallic acid and glucose (da Costa Souza et al. 2013). The prominent industrial uses of tannase are in the production of beverages and gallic acid, in disinfecting up the leather production waste matter bearing the noxious tannin and in the lessening of antinutritional consequences of tannins in animal nourishment (Mukherjee and Banerjee 2006).

Amongst South American countries, Brazil is well known for its wealth of caves, which are distributed in quite a few geologic patterns. Da Costa Souza et al. (2013) monitored and characterised filamentous fungal strains from the caves situated in some Brazilian states for production of tannase. Five hundred and forty-four fungal strains were characterised by the investigators; amongst them, 386 isolates had the capability to multiply in plates substituted with tannic acid medium as the only carbon supply. An overall of 32 strains were regarded by the investigators to be excellent producers of tannase; morphological identification revealed 20 *Aspergillus* and 12 *Penicillium* species. The investigators reported that the highest tannase production was attained in submerged fermentation by *Aspergillus japonicus* 246A (16.45 U/mg) and *A. tamarii* 3 (12.95 U/mg).

1.4.4.5 Urease-Producing Bacteria

Microbially induced calcite precipitation (MICP), a natural phenomenon associated with a wide range of bacterial species in an alkaline environment rich in Ca^{2+} (Achal and Pan 2011), is the resultant of complex biochemical reactions often governed by an enzyme urease (urea amidohydrolase; EC 3.5.1.5) produced by microorganisms (Zhang et al. 2015). It is used in civil and geotechnical engineering to improve the mechanical properties of soil for construction and environmental purposes by enhancing the strength and stiffness properties of soil through microbial activity and products (Ivanov and Chu 2008). The reported studies mainly used *Sporosarcina pasteurii* as the preferred urealytic bacteria for MICP process because it is non-pathogenic and has quick capability to produce urease (Cuzman et al. 2015).

Three bacterial isolates were selected by Omoregie et al. (2016) based on their quick urease production from Sarawak limestone caves using the enrichment culture technique. Urease activity of the bacterial isolates was measured by the investigators through changes in conductivity in the absence of calcium ions. The bacterial isolates showed an average urease activity of 13.33 mM urea hydrolysed/min. To summarise, their work suggested that the bacterial isolates investigated in their study could serve as constitutive producers of urease, thus useful in biocementation.

1.4.5 Antimicrobial and Anticancer Activities of Cave Microbes

Cervimycins A–D and xiakemycin A are the new antibiotics produced by bacteria characterised from caves (Herold et al. 2005). Herold et al. (2005) observed that these antibiotics are active against methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant *Enterococcus faecalis*. These compounds, besides being cytotoxic to cancer cells, also displayed antifungal activities. Some of the bioactive substances produced by cave microorganisms, the structures of which have not yet been determined, have a limited range of activity. For instance, some genera showed evidence of antifungal activity only against *Rhodotorula mucilaginosa*, whereas others exhibited merely anticancer activity against oral cavity and human small cell lung cancer cells (NCI-H187) [Tomova et al. 2013]. Some others only showed

antibacterial activities (Ningthoujam et al. 2009). It has been observed that some metabolites (e.g. xiakemycin A) produced by cave-dwelling microbes exhibited antibacterial, antifungal and cytotoxic activities (Jiang et al. 2015). Ghosh et al. (2017), however, have reported selective activity. For instance, bacterial strains like *Bacillus licheniformis* strain A12, *Pseudomonas fluorescens* strain B3 and *Sphingobacterium* sp. strain B7 showed activity only against Gram-positive but not against Gram-negative bacteria.

1.4.5.1 Antimicrobial Activities of Microbes from Few Selected Caves Globally

Klusaite et al. (2016) aspired to execute the foremost investigation of antimicrobial property of bacteria isolated from Krubera-Voronja Cave, Georgia, mostly looking at their action against pathogenic and non-pathogenic Gram-positive-bacteria. The detailed study of bioactive compounds of two Gram-positive strains (belonging to the family *Bacillaceae* in phylum *Firmicutes*) revealed diverse combinations of unstable compounds with antibacterial property. The key antibacterial compounds produced by the strains were pyrrolopyrazines pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl), pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(phenylmethyl) and 1,2-benzenedicarboxylic acid, bis(2-methylpropyl) ester. The fusion of the volatile antimicrobial compounds of both strains was antagonistic against non-pathogenic Gram-positive strains, while their activity against pathogenic Gram-positive bacteria substantially varied.

The Azorean lava tubes of Portugal have surroundings of intense geochemical situations where microorganisms must build up precise metabolic versions in order to multiply. Varela et al. (2009) sampled and screened one lava tube of Terceira Island in the Azores to study microorganisms having antibacterial activity. Several strains have been isolated, which suppressed food-borne human pathogens like *Salmonella typhimurium*, *Escherichia coli*, *Staphylococcus aureus* and *Listeria monocytogenes*.

The actinomycetes isolated from speleothems and soil samples of 19 karstic caves of Turkey were examined for antibiotic production in batch culture by Yücel and Yamaç (2010). Yücel and Yamaç (2010) studied the antimicrobial activity of the extracted active compound that showed bacteriostatic or bactericidal activity. The compound showed dose-dependent antimicrobial activity against antibiotic-resistant clinical bacterial strains, i.e. methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant *Enterobacter faecium* (VRE) and *Acinetobacter baumannii*. The MIC and the minimum bactericidal concentrations of the compound, however, were lower (125 μg/ml and 250–1000 μg/ml, respectively) than the standard antibiotic streptomycin.

Actinomycete strains isolated by Kay et al. (2013) in Falls Cave in Wells Gray Provincial Park, British Columbia, Canada, were used to treat American foulbrood disease (AFB) of honeybees (*Apis mellifera* L.) caused by *Paenibacillus larvae* (Neuendorf et al. 2004). The pathogen causes infection in the gut of honeybee larvae leading to death of bees during the larval stage. The strains screened by the investigators exhibited prominent antibacterial action against *P. larvae* with a

highest activity on day 4 of incubation at pH 7. Chemotaxonomic and phylogenetic analysis supported on 16S rRNA gene sequence showed that the strain was a constituent of the genus *Streptomyces*. According to the investigators, this is the foremost information on bioprospecting in volcanic caves of Canada.

1.4.5.2 Anti-MRSA (Methicillin-Resistant *Staphylococcus aureus*) and Antitubercular Activity

Radhakrishnan et al. (2014) studied the anti-MRSA and antitubercular activities of *Streptomyces* sp. isolated from the soil of Borra Caves, Visakhapatnam, India. The isolated strain generated pigment and showed bioactivity yet in low nutrient concentration (1/10X) YEME agar. This is the foremost report on antitubercular pigment from actinobacteria isolated from Indian caves. Based on the findings, the investigators concluded that Borra Caves are a budding resource for harvesting actinobacterial compounds. The strain isolated by the investigators will be a possible resource for bioactive pigments against MRSA and MDR (multi-drug-resistant) *M. tuberculosis*.

1.4.5.3 Anticancer Activity of Actinomycetes from Caves in Thailand

Nakaew et al. (2009) for the first time reported isolation of two very rare genera of actinomycetes, i.e. *Spirillospora* and *Nonomuraea*, from soil samples of the Phatup Cave Forest Park and Phanangkhoi Cave in northern Thailand. Two isolates showed antibacterial activity against Gram-positive bacteria besides cytotoxic activity.

1.5 Conclusions and Future Prospectives

There is a necessity of discovery of novel antibiotics. Industrial breakthrough programmes have basically dried out, and the utmost need of the hour is to go back to the pre-antibiotic age. The present chapter highlights the unexplored microbial diversity of caves that could be a vast supply of future bioactive compounds. The latest scientific advancements such as DNA sequencing and bioinformatics have showed the path that the hypogean environments might offer innovative approaches for the discovery of compounds hitherto unknown to the world. The sophisticated chemical partition and identification methods and manufacture of libraries of bioactive compounds from cave microbes for curative uses would be anticipated in the near future.

It is by and large taken into consideration that the microbial parvome grasps the guarantee for unearthing of fresh antibacterial medicines (Roemer et al. 2012). Increasing the fraction of culturable microorganisms would improve the possibility of discovery of new active metabolites (Zhang 2005). Many of these metabolites showed activity towards a few familiar antibiotic-resistant microbial strains (Nimaichand et al. 2015). Caves are a dynamic reservoir of potential antimicrobials; therefore, exploring cave microbiota might open up new horizons for drug discovery. This is even more relevant considering that all the antagonistic activities of bacteria were divulged by cultivating bacteria; thus there is a reasonable possibility

that 99% of the prospective microorganisms from environmental samples have been missed (Mandal et al. 2014), as majority of them are defiant to laboratory culture (Sharma et al. 2005). It is very well known that a mere 0.1–1% of the microorganisms which colonise the biosphere have been grown under laboratory conditions (Zhang 2005). The relatively trifling pace of accomplishment these methods have encountered in the preceding few decades can be described, by the deficiency of logical management of the habitats taken into consideration (Jones et al. 2014), which has led to indiscriminate sampling and neglect of the authentic prospects of these habitats (Harvey 2000).

The majority of the studies have been performed based on cultivation-based methods, even though a handful of investigators have also employed metagenomics. As of now, the execution of metagenomics for the examination of genes from cave environments is in the early stages. The application of metagenomics in retrieving novel genes from cave metagenomes for antimicrobials is expected to yield promising results in the future.

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2 Diversity of Nitrogen-Fixing Symbiotic Rhizobia with Special Reference to Indian Thar Desert

Nisha Tak and Hukam Singh Gehlot

Abstract

The symbiotic nitrogen-fixing bacteria are found in diverse climatic conditions and are ecologically important. The classification of rhizobia has always been fascinating; with the advent of polyphasic approaches, it is continuously changing by addition of new genera and species and the reclassification and discovery of nontraditional rhizobia. In comparison with crop legumes, the study of symbiotic associations in wild/native legumes has led to the discovery of several genetically diverse rhizobia. In the era of global climate change, increasing desertification, and for food security, the identification and characterization of rhizobia adapted to arid and hot climatic conditions are important. With this aim desert rhizobia associated with several native legumes belonging to different tribes have been broadly studied from less-explored regions of Indian Thar Desert. The diverse legumes in alkaline soils of the Thar Desert are found to be nodulated by traditional rhizobial genera, *Ensifer* and *Bradyrhizobium.* On the basis of core gene phylogeny, the *Ensifer* strains affiliated to mimosoid, cesalpinioid, and papilionoid legumes clustered into novel clades and lineages. *Bradyrhizobium* strains phylogenetically diversified from the *B. yuanmingense* type strain are microsymbiont of species of *Tephrosia*, *Alysicarpus*, *Crotalaria*, and *Chamaecrista* in addition to strains of *Ensifer*. The tree rhizobia (isolated from *Vachellia*, *Senegalia*, *Prosopis*, *Mimosa*) have host range restricted to tree species and therefore could be used as an inoculum in forestry practices. The other native rhizobia isolated from wild legumes (*Tephrosia* and *Chamaecrista*) are compatible with crop legumes (*Vigna*, *Cyamopsis*, *Glycine max*) and can be useful in preparation of consortia for extension of agricultural practices.

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Keywords
Root nodule bacteria · *Ensifer* · *Bradyrhizobium* · Wild legumes · Thar Desert

2.1 Introduction

The ability to fix atmospheric nitrogen to ammonia is exclusively confined to prokaryotic organisms (diazotrophs) that contain the nitrogenase enzyme complex and the phenomenon is known as biological nitrogen fixation (BNF). Diazotrophs can be broadly divided into two groups: (i) free-living/nonsymbiotic and (ii) mutualistic/symbiotic. Eukaryotic organisms are incapable to fix nitrogen, and therefore few of them establish a symbiotic relationship with diazotrophic bacteria. In these interactions, eukaryotic organisms supply nutrients and energy to the diazotrophs in exchange for fixed nitrogen. Legume-rhizobia symbiosis is one of such plant-microbe interactions that contribute fixed nitrogen to the biosphere in a cost-effective and eco-friendly manner. The symbiotic diazotrophs invade or colonize the root (and occasionally stem) of the host plant, where they multiply and induce nodule organogenesis. Rhizobia are heterotrophic, aerobic, non-sporulated, gram-negative, and rod-shaped nitrogen-fixing bacteria that have coevolved with host legumes (Sprent 2001). Rhizobia are widely distributed in diverse geographical and ecological niches of the world. Besides diazotrophy, a group of rhizobia have been discovered which show methylotrophy, for example, *Methylobacterium* strains isolated from species of *Crotalaria* (Sy et al. 2001), and stem-nodulating photosynthetic bradyrhizobia have been isolated from species of *Aeschynomene* (Molouba et al. 1999). The rhizobia are among the most extensively studied group of bacteria due to their nitrogen-fixing ability and could replace synthetic nitrogen fertilizers, in nitrogen-poor soils (Zahran 2001). Relatively little is known about the nitrogen-fixing value of most of the wild legume species found in arid conditions (Sprent 2001). Rhizobia associated with wild/native legumes establish effective symbiosis under harsh environmental conditions and are more tolerant to abiotic stresses (salinity, alkalinity, drought, elevated temperatures, etc.) than the crop rhizobia (Zahran 2001). Thus symbiotically efficient rhizobia with increased tolerance to high salt, pH, and temperature could enhance the production of food and forage legumes in semiarid and arid regions of the world. There are many potentially nodulated legumes native to arid and semiarid areas worldwide which need to be explored for their microsymbiont diversity (Sprent and Gehlot 2010; Panwar et al. 2014).

All bacteria isolated from root nodules were initially classified into the genus *Rhizobium* until the early 1980s. Later two distinct groups of rhizobia were recognized based on growth on culture medium: fast- and slow-growing. The fast-growing rhizobia are acid-producing and associated mainly with temperate legumes, while the slow-growing (bradyrhizobia) are associated with tropical and subtropical legumes. For a long time, the grouping of root nodule bacteria was based on nodulation with certain host plants called as the cross-inoculation group. The Bergey's manual has played a fundamental role in rhizobial taxonomy. Development in

molecular biology techniques and with the advent of numerical taxonomy considering wide range of characteristics led to the definition of new genera and species and also the renaming of some species (Jordan 1982; Graham et al. 1991; Young et al. 2003). Moulin et al. (2001) published in Nature the nodulation of legumes by *Burkholderia*, a genus that belongs to class beta-proteobacteria. Sawada et al. (2003) reported 44 bacterial species distributed in 12 genera, 10 of which belong to the class "alpha-proteobacteria" (*Allorhizobium*, *Azorhizobium*, *Blastobacter*, *Bradyrhizobium*, *Devosia*, *Mesorhizobium*, *Methylobacterium*, *Ochrobactrum*, *Rhizobium*, and *Ensifer*) and 2 to the class "beta-proteobacteria" (*Burkholderia* and *Cupriavidus*). Peix et al. (2015) reviewed that there are 15 rhizobial genera belonging to proteobacteria under "classical rhizobia" (*Azorhizobium*, *Bradyrhizobium*, *Mesorhizobium*, *Rhizobium*, and *Ensifer*) and "new rhizobia" (*Aminobacter*, *Burkholderia*, *Cupriavidus*, *Devosia*, *Herbaspirillum*, *Methylobacterium*, *Microvirga*, *Ochrobactrum*, *Phyllobacterium*, and *Shinella*) with nodulating and non-nodulating species. Shamseldin et al. (2017) mentioned about 18 genera of rhizobia with more than 250 species belonging to class alpha- and beta-proteobacteria. At the time of writing this book chapter, the following legume-nodulating genera are known: *Rhizobium*, *Neorhizobium*, *Pararhizobium*, *Ensifer*, *Shinella*, *Mesorhizobium*, *Phyllobacterium*, *Aminobacter Bradyrhizobium*, *Blastobacter*, *Photorhizobium*, *Devosia*, *Azorhizobium*, *Methylobacterium*, *Microvirga*, and *Ochrobactrum* in alpha-proteobacteria and *Paraburkholderia* (Sawana et al. 2014), *Cupriavidus*, *Trinickia*, and *Herbaspirillum* in beta-proteobacteria (List of Prokaryotic Names with Standing in Nomenclature (LPSN); http://www.bacterio.net).

2.2 Diversity of Classical and Unconventional Rhizobia

Rhizobial systematics is becoming complex and rapidly changing, and recently many new species have been recognized. The term "root nodule bacteria" (RNB) is nowadays used for all groups of bacteria that have been isolated from root nodule; they may be nodulating or non-nodulating. The legume-nodulating bacterial species within class alpha-proteobacteria comprises of six families, namely, *Bradyrhizobiaceae*, *Brucellaceae*, *Hyphomicrobiaceae*, *Methylobacteriaceae*, *Phyllobacteriaceae*, and *Rhizobiaceae*, under the order *Rhizobiales* and families *Burkholderiaceae* and *Oxalobacteraceae* within the order *Burkholderiales* in class beta-proteobacteria. In addition to classical and new rhizobia, there are few reports from the class gamma-proteobacteria (Benhizia et al. 2004; Shiraishi et al. 2010). Non-rhizobial root nodule endophytes belonging to gamma-proteobacteria (*Enterobacter*, *Klebsiella*, *Pantoea*, *Pseudomonas*, *Stenotrophomonas*), *Actinobacteria* (*Arthrobacter*, *Brevibacterium*, *Microbacterium*, *Micromonospora*, *Mycobacterium*, *Streptomyces*), *Firmicutes* (*Bacillus*, *Fontibacillus*, *Paenibacillus*), and *Sphingobacteria* (from *Clitoria ternatea*) have also been isolated from many indigenous legumes (Hoque et al. 2011; Aserse et al. 2013; de Meyer et al. 2015; Boukhatem et al. 2016). Martínez-Hidalgo and Hirsch (2017) reviewed the

existence of many non-rhizobial root nodule endophytes, many of them are nitrogen fixers and few of them were able to induce the formation of nodules. Biogeography of nodulated legumes and associated nitrogen-fixing microsymbionts was reviewed by Sprent et al. (2017) in terms of both longitudinal and latitudinal trends. Our knowledge about rhizobial diversity associated with nodulated legumes is limited due to culture-based approaches used to characterize the strains. The root nodules are house for many conventional and unconventional rhizobia. In this chapter we emphasized mainly the diversity of rhizobial genera belonging to class alpha- and beta-proteobacteria and some recent changes made in classification.

2.2.1 Alpha-Proteobacteria

2.2.1.1 Rhizobiaceae

Family *Rhizobiaceae* comprises of more than 120 sp. that are distributed into the following genera: *Agrobacterium*, *Allorhizobium*, *Ciceribacter*, *Ensifer*, *Neorhizobium*, *Rhizobium*, and *Shinella* (Mousavi et al. 2014). The largest genus *Rhizobium* is extremely heterogeneous and has more than 100 species, comprising of both nodulating and few non-nodulating strains. It has gone through major revision based on multilocus sequence phylogenetic analysis of housekeeping genes. The genus *Neorhizobium* encompasses the former species of *Rhizobium* (*R. alkalisoli*, *R. huautlense*, *R. vignae*, and *R. galegae*), and three new species combinations were described, namely, *N. galegae*, *N. huautlense*, and *N. alkalisoli* (Mousavi et al. 2014). The novel genus *Pararhizobium* recently described by Mousavi et al. (2015) comprised of four new species (*P. giardinii*, *P. capsulatum*, *P. herbae*, and *P. sphaerophysae*).

The genus *Ensifer* (formerly *Sinorhizobium*) (Chen et al. 1988) presently contains 21 species, of which 19 have been validly published based on polyphasic studies. Most of the species have been isolated from root nodules of *Glycine max* (for details of species, see the review by Shamseldin et al. 2017) and tree species from the African continent (de Lajudie et al. 1994; Nick et al. 1999). Few strains (*E. meliloti* and *E. medicae*) nodulate specifically medics, melilots, and spp. of *Trigonella* (de Lajudie et al. 1994; Rome et al. 1996; El Batanony et al. 2015; Gaur et al. 2018) in different parts of the world. From the New World, species like *E. americanus* (from *Acacia acatlensis*) (Toledo et al. 2003) and *E. mexicanus* (*Acacia angustissima*) (Lloret et al. 2007) have been reported. Two species, namely, *E. abri* and *E. indiaense*, isolated from tropical legumes, *Abrus precatorius* and *Sesbania rostrata*, respectively, have been published from India (Ogasawara et al. 2003), but they have not yet been included in the Validation List of the International Journal of Systematic and Evolutionary Microbiology, and no type strains have been submitted so far at international culture collections. Recently new species *E. aridi* (not validly published) has been reported from hot-arid regions of three continents (Asia, Africa, and America). The reported strains of *E. aridi* isolated from different legume host were genetically similar but harbored different *sym* genes (Le Queré et al. 2017).

Ensifer sojae has been isolated from root nodules of *G. max* grown in saline-alkaline soils of China (Li et al. 2011). Similarly, Li et al. (2016) reported genetically diverse rhizobia nodulating *Sesbania cannabina* in saline-alkaline soils. Cheng et al. (2002) observed that the nodulation response of *Medicago sativa* and *Medicago murex* differed with soil acidity.

The genus *Allorhizobium* described by de Lajudie et al. (1998) contains one species (*A. undicola*) which effectively nodulates *Neptunia natans* in Senegal. The merging of the three genera (*Agrobacterium*, *Rhizobium*, and *Allorhizobium*) was done into a single genus, *Rhizobium*. On the basis of 16S rRNA phylogenetic analyses, four species of *Agrobacterium* (*A. tumefaciens*, *A. radiobacter*, *A. rhizogenes*, and *A. vitis*) were transferred to the genus *Rhizobium* (Young et al. 2003). Bacteria related to *Agrobacterium* were identified among the root nodules of several tropical leguminous plants from Africa (de Lajudie et al. 1999; Mhamdi et al. 2005). Nodulation and nitrogen-fixing genes were not detected in these strains, and it has been confirmed that these *Agrobacterium*-like strains enter the nodules by mixed infection with a rhizobia capable of inducing nodule resulting in mixed population within the nodule (Mhamdi et al. 2005).

The genus *Shinella* has one species (*S. kummerowiae*), a symbiotic bacterium isolated from root nodules of the herbal legume *Kummerowia stipulacea* grown in Shandong province of China (Lin et al. 2008). Newly described genus, *Ciceribacter* (*C. lividus*), was isolated from rhizosphere soil of *Cicer arietinum* from Kannivadi, India (Kathiravan et al. 2013).

2.2.1.2 Phyllobacteriaceae

As per Shamseldin et al. (2017), family *Phyllobacteriaceae* comprises about 49 nitrogen-fixing species within three genera, namely, *Mesorhizobium* (40 species), *Phyllobacterium* (8 species), and *Aminobacter* (1 species). The genus *Mesorhizobium* was described by Jarvis *et al.* (1997) with the aim to reassign some species previously included in the *Rhizobium* genus. Several *Rhizobium* species were transferred to this genus (*M. loti*, *M. huakuii*, *M. ciceri*, *M. tianshanense*, *M. mediterraneum*). *Mesorhizobium* is a wide spread rhizobial genus nodulating a wide range of legumes in addition to *Cicer arietinum* growing mainly in acidic soils (Peix et al. 2015). However, Zhang et al. (2012) suggested that mesorhizobia are the preferred microsymbionts of chickpea growing in alkaline soils of northwest China.

Genus *Phyllobacterium* was proposed to accommodate bacterial species isolated from leaf nodules of members of Rubiaceae (*Pavetta*, *Psychotria*, and *Sericanthe*) and Myrsinaceae (*Ardisia*) in tropical continental Africa and Asia (Knösel, 1984). *Chromobacterium lividum* has also been reported from the leaf-nodulated members of these families. *Phyllobacterium trifolii* was isolated from root nodules of *Trifolium pratense* growing in a Spanish soil (Valverde et al. 2005). It harbors symbiotic genes and infectivity tests experiments with this species revealed that it forms nodules on the roots of *Trifolium repens* and *Lupinus albus* (Valverde et al. 2005). Recently *P. loti* was isolated from nodules of *Lotus corniculatus* (Sánchez et al. 2014).

Maynaud et al. (2012) described *Aminobacter anthyllidis*, a metal-resistant bacteria nodulating *Anthyllis vulneraria* a legume host suitable for phytostabilization in mining areas.

2.2.1.3 Bradyrhizobiaceae

Family *Bradyrhizobiaceae* contains three nitrogen-fixing genera *Bradyrhizobium*, *Blastobacter*, and *Photorhizobium*. The genus *Bradyrhizobium* was created to accommodate slow-growing bacteria capable of establishing nitrogen-fixing symbioses with a broad range of plants belonging to three subfamilies of the family Leguminosae and characterized by an alkaline reaction in culture media. The genus *Bradyrhizobium* presently includes more than 40 species isolated from the nodules of highly divergent legume tribes, including herbaceous and woody species of tropical and temperate origin (Sprent et al. 2017; Shamseldin et al. 2017). Jordan (1982) reported *B. japonicum* from root nodules of *Glycine max*, and since then many species (*B. daqingense*, *B. huanghuaihaiense*, *B. liaoningense*, and *B. ottawaense*) have been isolated which are associated with soybean. Bradyrhizobial species have also been reported as microsymbionts of *Arachis hypogaea* (*B. arachidis*, *B. guangxiense*, *B. guangdongense*, and *B. subterraneum*) and *Vigna unguiculata* (*B. kavangense*, *B. brasilense*, *B. vignae*, and *B. manausense*). Type strain *B. canariense* is an acid-tolerant endosymbiont that effectively nodulates shrubs of the tribes Genisteae and Loteae (Vinuesa et al. 2005). Strain *B. iriomotense* was isolated from a tumor-like root of the legume *Entada koshunensis* from Japan (Islam et al. 2008). Ramírez-Bahena et al. (2009) described two species (*B. pachyrhizi* and *B. jicamae*) that nodulate *Pachyrhizus erosus*. From Morocco (Africa) *B. cytisi* was isolated from nodules of *Cytisus villosus* and *B. retamae* from *Retama monosperma* (Chahboune et al. 2011).

The type strains of *Bradyrhizobium* on the basis of housekeeping genes phylogeny clusters into two mega clades (Ojha et al. 2017). *Bradyrhizobium* strains belonging to two mega clades do not show a particular geographical pattern as observed for type strains of *Ensifer* (Sankhla et al. 2017); instead they have been found intermingled in both clades representing different continents from where bradyrhizobia have been reported. Mega clade-I contains several Asian species (*B. arachidis*, *B. daqingense*, *B. ganzhouense*, *B. guangxiense*, *B. guangdongense*, *B. huanghuaihaiense*, *B. iriomotense*, *B. japonicum*, *B. liaoningense*, and *B. yuanmingense*), African species (*B. cytisi*, *B. kavangense*, *B. rifense*, *B. subterraneum*, and *B. vignae*), and few species from Europe (*B. betae* and *B. canariense*). From the New World, species such as *B. americanum*, *B. centrosemae*, *B. centrolobii*, *B. forestalis*, *B. ingae*, *B. manausense*, *B. neotropicale*, *B. ottawaense*, and *B. stylosanthis* have been reported. The mega clade-II also contains few Asian (*B. erythrophlei*, *B. ferriligni*, and *B. lablabi*), African (*B. namibiense* and *B. retamae*), and European (*B. valentinum*) species. Mega clade-II is mainly represented by species isolated from the New World such as *B. brasilense*, *B. elkanii*, *B. embrapense*, *B. icense*, *B. jicamae*, *B. mercantei*, *B. macuxiense*, *B. pachyrhizi*, *B. paxllaeri*, *B. tropiciagri*, and *B. viridifuturi*.

Symbiotic *Bradyrhizobium* strains have also been isolated from the nodules of non-legume plants (*Trema aspera*) growing between rows of tea in the Pangia district of New Guinea (Trinick 1973). The discovery of photosynthetic *Bradyrhizobium* strains that can induce nitrogen-fixing nodules on stems of the legume *Aeschynomene* was made (Molouba et al. 1999). The microsymbionts of *Aeschynomene indica* are unique as they form nodules on plant stems, branches, and roots; and some of them produce the photosynthetic pigments. The genus *Photorhizobium* contains only a single photosynthetically active species *P. thompsonianum* efficiently nodulating *A. indica* (Eaglesham et al. 1990). The aquatic budding bacterium *Blastobacter denitrificans* also forms nitrogen-fixing symbioses with *A. indica* (van Berkum and Eardly 2002).

2.2.1.4 Hyphomicrobiaceae

Family *Hyphomicrobiaceae* includes two nodulating and nitrogen-fixing genera *Devosia* and *Azorhizobium*. The genus *Azorhizobium* described by Dreyfus et al. (1988) includes nitrogen-fixing root and stem-nodulating microsymbiont species that can also fix nitrogen *ex planta* under micro-aerobic conditions. Genus *Azorhizobium* contains three species; two of them effectively fix nitrogen with species of *Sesbania*. *Azorhizobium caulinodans* was isolated from the stem nodules of *Sesbania rostrata* from Africa (Dreyfus et al. 1988), and *A. doebereinerae* is microsymbiont of *Sesbania virgata* in Brazil (de Souza Moreira et al. 2006). The unique feature of *A. caulinodans* is that it fixes nitrogen both in aerobic cultures and in micro-aerobic symbiosis with its legume host *S. rostrata*. Recently strain *A. oxalatiphilum* was isolated from macerated petioles of *Rumex* sp. through enrichment in mineral medium.

The genus *Devosia* has single nodulating species, *D. neptuniae*, identified by Rivas et al. (2003) that efficiently nodulates aquatic legume *Neptunia natans* in India. This strain through horizontal transfer has acquired symbiotic genes from a broad host range strain, *Rhizobium tropici* (Rivas et al. 2003).

2.2.1.5 Methylobacteriaceae

Family *Methylobacteriaceae* comprises of two rhizobial genera *Methylobacterium* and *Microvirga* with three and four species, respectively, that can induce nitrogen-fixing nodules on roots of legume plant. The genus *Methylobacterium* includes pink-pigmented facultative methylotrophic (PPFM) bacteria that are strictly aerobic and able to grow on one-carbon compounds such as formate, formaldehyde, and methanol as sole carbon source. Sy et al. (2001) reported facultative methylotrophic species forming nodules in the *Crotalaria* species. The type strain *M. nodulans* was isolated from *C. podocarpa* from Senegal (Jourand et al. 2004). *Methylobacterium* strains within the root nodules obtain carbon from photosynthates of host plant as well as from methylotrophy. Madhaiyan et al. (2006) isolated several nodulating and plant-growth promoting *Methylobacterium* species from tropical legumes.

Ardley et al. (2012) reported three species of *Microvirga*, namely, *M. lupini*, isolated from nitrogen-fixing nodules of *Lupinus texensis* in Texas, and *M. lotononidis* and *M. zambiensis* from *Listia angolensis* collected in Zambia.

2.2.1.6 Brucellaceae

The family *Brucellaceae* contains fast-growing, nitrogen-fixing strains in the genus *Ochrobactrum*, which has two species, namely, *O. lupini* and *O. cytisi*, isolated from the nodules of *Lupinus honoratus* and *Cytisus scoparius*, respectively (Trujillo et al. 2005; Zurdo-Piñeiro et al. 2007).

2.2.2 Beta-Proteobacteria

2.2.2.1 Burkholderiaceae

Family *Burkholderiaceae* comprises of the two genera *Burkholderia sensu lato* and *Cupriavidus* (former *Ralstonia*). Genus *Burkholderia sensu lato* is a big and complex group including diverse species with different physiological and ecological properties; pathogenic strains have been isolated from animals and humans; some are phytopathogenic and others are beneficially associated with plant (root nodule symbionts and nonsymbiotic strains) and have also been isolated from a very wide range of environmental habitats (soil and water) [Gyaneshwar et al. 2011]. Taxonomy of *Burkholderia sensu lato* has undergone major changes several times based on concatenated phylogenetic analysis of housekeeping gene fragments and whole genome sequences. Sawana et al. (2014) based on molecular signatures and phylogenomic analysis proposed for the division of *Burkholderia sensu lato* containing pathogenic organisms, and a separate new genus *Paraburkholderia* was proposed for harboring environmental species. Eleven species of the genus *Burkholderia* were later transferred to genus *Paraburkholderia*, and a new genus *Caballeronia* was proposed to accommodate 12 species of the genera *Burkholderia* and *Paraburkholderia* (Dobritsa and Samadpour, 2016). Presently this complex group of *Burkholderia sensu lato* is split into the following genera: *Burkholderia sensu stricto* (includes the human and animal pathogens), *Caballeronia* (includes the plant beneficial and environmental strains), and *Paraburkholderia* (includes the nitrogen-fixing legume microsymbionts) (Sawana et al. 2014; Dobritsa and Samadpour 2016). Based on comparative genomic analysis recently, Estrada-de los Santos et al. (2018) described two novel genera *Mycetohabitans* and *Trinickia* which form two distinct and unique clades, including two (*M. endofungorum* and *M. rhizoxinica*) and four (*T. caryophylli*, *T. dabaoshanensis*, *T. soli*, and *T. symbiotica*) new combinations of species.

Initially two legume-nodulating *Burkholderia* (*sensu lato*) strains, *P. tuberum* STM678^T and *P. phymatum* STM815^T, were isolated from root nodules of *Aspalathus carnosa* and *Machaerium lunatum*, respectively (Moulin et al. 2001). Presently, the following legume-nodulating species of genus *Paraburkholderia* have been described including *P. aspalathi*, *P. caballeronis*, *P. caribensis*, *P. diazotrophica*, *P. dilworthii*, *P. kirstenboschensis*, *P. mimosarum*, *P. nodosa*, *P. phenoliruptrix*, *P. piptadeniae*, *P. phymatum*, *P. tuberum*, *P. rhynchosiae*, *P. ribeironis*, *P. sabiae*, and *P. sprentiae* (Estrada-de los Santos et al. 2018). The following species of *Paraburkholderia* are root nodule symbionts of species of *Mimosa* (Estrada-de los Santos et al. 2018): *P. caribensis* (*M. pudica* and *M. diplotricha*); *P.*

diazotrophica (*Mimosa* spp.); *P. mimosarum* (*M. pigra* and *M. pudica*); *P. nodosa* (*M. bimucronata* and *M. scabrella*); *P. phenoliruptrix* (*M. flocculosa*); *P. phymatum* (*M. pudica*); and *P. sabiae* (*M. caesalpiniifolia*). Other beta rhizobia strains (*C. taiwanensis* and *T. symbiotica*) belonging to different genera are specifically symbionts of species of *Mimosa*. Two newly described species *P. piptadeniae* and *P. ribeironis* nodulates piptadenia group (*Piptadenia gonoacantha*, tribe mimoseae) in Brazil (Bournaud et al. 2013, 2017). The following species of *Paraburkholderia* nodulates papilionoids (Estrada-de los Santos et al. 2018): *P. sprentiae* (isolated from *Lebeckia ambigua* root nodules); *P. rhynchosiae* (from root nodules of *Rhynchosia ferulifolia*); *P. dilworthii* (from *Lebeckia ambigua* root nodules); *P. kirstenboschensis* (nodulates papilionoid legumes *Virgilia oroboides*, *Hypocalyptus coluteoides*, *H. oxalidifolius*, and *H. sophoroides* indigenous to South Africa); and *P. tuberum* (nodulates several *Cyclopia* species). *Paraburkholderia* type strains (*P. phymatum* and *P. nodosa*) belonging to mimosoid clade also nodulate the promiscuous papilionoid (*Phaseolus vulgaris*, *Vigna unguiculata*, and *Macroptilium atropurpureum*) legumes of tribe Phaseoleae, whereas the *T. symbiotica* strain has restricted host range (*Mimosa* spp.) and failed to nodulate these promiscuous legumes. Type strain *P. caballeronis* originally isolated from tomato has the ability to effectively nodulate *P. vulgaris* (Martínez-Aguilar et al. 2013). Type strain *P. aspalathi* was isolated from root nodules of the South African legume *Aspalathus abietina* (tribe Crotalarieeae). The legume-nodulating *Paraburkholderia* strains of class beta-proteobacteria possess nodulation genes phylogenetically related to those found in legume symbionts of the class alpha-proteobacteria suggesting that the beta rhizobia have evolved through lateral gene transfers (Chen et al. 2003).

Genus *Cupriavidus* presently contains two rhizobial species, *C. taiwanensis* nodulating *Mimosa* sp. which has been repeatedly isolated from root nodules on the pan-tropical weeds *M. pudica* and *M. diplotricha* in Taiwan (Chen et al. 2001, 2003) and also from India (Gehlot et al. 2013). Another species is *C. necator* that was isolated through trap (in soils of Brazil) experiments from root nodules of two promiscuous legume species, *P. vulgaris* and *Leucaena leucocephala* (da Silva et al. 2012). This species can effectively nodulate other promiscuous legumes such as *M. atropurpureum*, *V. unguiculata*, and *M. caesalpiniaefolia* (da Silva et al. 2012).

2.2.2.2 Oxalobacteraceae

The genus *Herbaspirillum* in family *Oxalobacteraceae* within the order *Burkholderiales* was first described to include bacterial strains associated with roots of several cereals. Valverde et al. (2003) reported a nitrogen-fixing bacterium *H. lusitanum* associated with root nodules of *P. vulgaris* plants grown in soil from Portugal. Genus *Herbaspirillum* and another genus *Variovorax* of the beta-proteobacteria have been isolated from root nodules of species of *Acacia* (*A. salicina* and *A. stenophylla*) from Southeastern Australia (Hoque et al. 2011). It has also been isolated from nodules of *Aspalathus linearis*, but nodulation has not been confirmed (Hassen et al. 2012).

2.2.3 Gamma-Proteobacteria

Besides nodulating bacteria from alpha- and beta-proteobacteria, there is a report of gamma-proteobacteria nodulating *Hedysarum* sp. (Benhizia et al. 2004). *Enterobacter cloacae* and *E. kobei* were isolated from root nodules of the three species of *Hedysarum* (*H. carnosum*, *H. spinosissimum*, and *H. pallidum*) growing in native stands in different habitats in Algeria (Benhizia et al. 2004). Muresu et al. (2008) concluded there are frequent reports of coexistence of symbiotic culturable rhizobia with non-culturable rhizobia and other endophytic bacterial taxa (of the order *Enterobacteriales* or *Pseudomonadales*) within root nodules suggesting that diversity of bacterial species nodulating legumes may be broader than expected. Shiraishi et al. (2010) noted the ability of *Pseudomonas* sp. to nodulate *Robinia pseudoacacia*. These gamma-proteobacteria strains probably acquired the symbiotic genes from symbiotic-rhizobial species in the soil and rhizosphere through lateral gene transfer.

2.3 Genetic Diversity of Indian Thar Desert Rhizobia

Based on various criterions such as annual precipitation, temperature, soil, geography, and types of plant community, the deserts can be classified to hyper-arid, arid, and semiarid (Sprent and Gehlot, 2010). The Great Indian Desert or the Indian Thar Desert comes under low altitude arid and semiarid region with an area of about 200,000 km^2. It is a subtropical desert (https://www.britannica.com/place/Thar-Desert), and most of its part (61%) is present in Western Rajasthan which experiences an arid climatic regime (hot desert climate found under the subtropical ridge). The precipitation is low and varies from hyper-arid areas to semiarid areas, some areas have saline tracts, and soils are alkaline with low fertility. The native legumes of this region are subject to extremely stressful and harsh environmental conditions. In arid regions, heat stress affects both the free-living and symbiotic life of rhizobia, while desertification causes a negative impact on legume-rhizobia symbiosis. Still, a large number of legumes of Thar Desert belonging to different subfamilies were reported to be nodulated (Panwar et al. 2014, Gehlot et al. 2012). As questioned by Sprent and Gehlot (2010) “Nodulated legumes in arid and semiarid environments: are they important?” the answer would be yes, these legumes that have coevolved with their rhizobial partners in the Thar Desert have immense potential for fixing atmospheric nitrogen, reforestation, in controlling soil erosion, and increasing the soil fertility. Sprent and Gehlot (2010) mentioned about some nodulating native, perennial drought-tolerant, and drought-escaping annual legumes from the Thar Desert. The rhizobia of wild legumes have novel genotype and better phenotype/traits than the homologous crop rhizobia. Therefore, isolation of effective and promiscuous rhizobia from wild legumes to inoculate other legume crops is a better strategy to improve the efficiency of the rhizobium-legume symbiosis.

In the era of global climate change and for food security, studies such as exploration, identification, and characterization of indigenous nitrogen-fixing microsymbiont associated with native medicinal and food crop legumes are needed. Such studies are more relevant when working on the microsymbionts specific to legumes growing in a particular soil type and climatic conditions. Earlier research on rhizobia-legume symbiosis was restricted to few agriculturally important food legume crops like soybean, common bean, cowpea, species of *Vigna*, and associated species. In the last one decade, several studies have been conducted on molecular characterization and analysis of phylogenetic diversity of root nodule bacteria associated with native/wild legumes (such as species of *Vachellia*, *Senegalia*, *Prosopis*, *Mimosa*, *Chamaecrista*, *Crotalaria*, *Alysicarpus*, *Rhynchosia*, *Tephrosia*, *Indigofera*, *Trigonella*, and *Vigna*) of Indian Thar Desert (Gehlot et al. 2012, 2013, 2014, 2016; Tak et al. 2013, 2016a, b; Panwar et al. 2014; Ojha et al. 2015; Sankhla et al. 2015, 2017, 2018; Choudhary et al. 2017, 2018; Rathi et al. 2017, 2018; Gaur et al. 2018). Panwar et al. (2014) reviewed the status of nodulation of more than 30 native legume species belonging to 3 subfamilies and more than 10 legume genera. Gehlot et al. (2012) reported that in the hot-dry and alkaline soils of Indian Thar Desert (Western Rajasthan), native legumes are nodulated by genetically diverse nitrogen-fixing *Ensifer*, *Bradyrhizobium*, and *Rhizobium* strains. The dominant microsymbionts of most legumes were species of *Ensifer* (Gehlot et al. 2012, 2013; Ojha et al. 2015; Sankhla et al. 2015, 2017, 2018; Tak et al. 2016a, b; Ardley 2017; Choudhary et al. 2017, 2018; Rathi et al. 2017, 2018; Gaur et al. 2018) and few novel *Ensifer* strains have been characterized at genomic level (Tak et al. 2013; Gehlot et al. 2016; Le Queré et al. 2017). Some native legumes including species of *Tephrosia*, *Chamaecrista*, and *Alysicarpus* in the Thar Desert are effectively nodulated by both *Ensifer* and *Bradyrhizobium* strains (Gehlot et al. 2012; Ojha et al. 2015; Tak et al. 2016b; Rathi et al. 2017, 2018).

2.3.1 Phylogenetic Diversity of Thar Desert *Ensifer* Strains

Genetically distinct groups of old-world *Ensifer* strains have evolved in the Thar Desert and are more adapted to stressed environment and dominating in the alkaline soils. The Thar-*Ensifer* strains are promiscuous and nodulating papilionoid, mimosoid, and caesalpinioid legumes. In the maximum likelihood phylogenetic tree of the Thar Desert *Ensifer* strains isolated from root nodules of various wild legumes *Vachellia jacquemontii* (AJ), *Vachellia leucophloea* (AL), *Senegalia senegal* (AS), *Crotalaria burhia* (CB), *Chamaecrista pumila* (CP), *Mimosa hamata* (MH), *Mimosa himalayana* (MHM), *Trigonella foenum-graecum* (TFG), *Tephrosia falciformis* (TF), *Tephrosia leptostachya* (TL), *Tephrosia purpurea* (TP), *Tephrosia villosa* (TV), *Tephrosia wallichii* (TW), and *Vigna trilobata* (VT) of Western Rajasthan based on 16S rRNA gene sequences (Fig. 2.1), the strains clustered into novel clades and lineages. Most of the strain isolated from mimosoid trees and shrubs (MH,

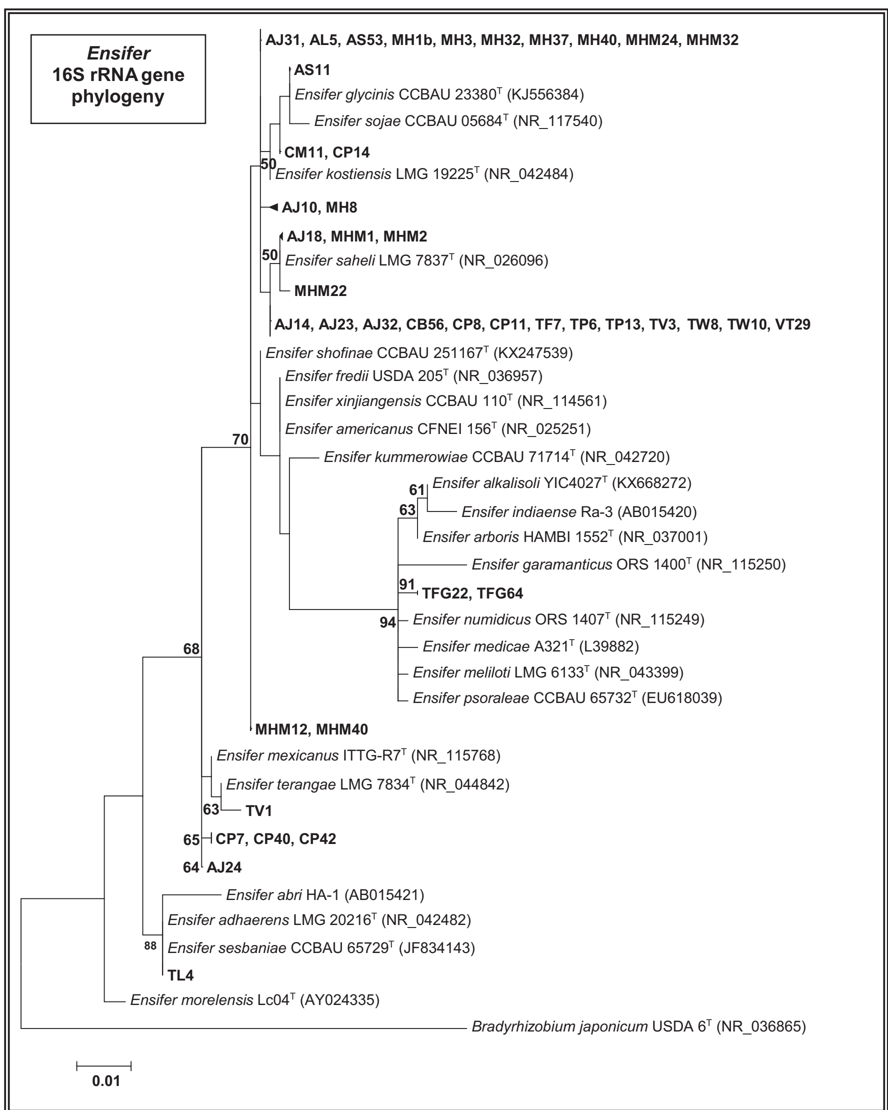

Fig. 2.1 Maximum likelihood phylogenetic tree of **Thar Desert *Ensifer*** strains isolated from root nodules of various wild legumes of Western Rajasthan based on **16S rRNA** gene sequences. Bootstrap values more than 50% calculated for 1000 replications are indicated at internodes. The scale bar indicates 1% nucleotide substitution per site. (Abbreviation: superscript T stands for type strain)

MHM, AJ, AS, AL), papilionoid shrubs/herbs (CB, TF, TP, TV, TW, VT), and caesalpinioid herb (CP) shared close similarity with *E. saheli* LMG 7837^T (*Sesbania cannabina*, Senegal) (de Lajudie et al. 1994). Few strains (TV1, CP7, CP40, CP42) shared close similarity with *E. terangae* LMG 7834^T (*Senegalia laeta*, Senegal) (de Lajudie et al. 1994). Strains CM11 and CP14 shared close affinity with another old-world *Ensifer*, *E. kostiensis* HAMBI 1489^T (*Senegalia senegal*, Sudan) (Nick et al. 1999). Strain AS11 clustered with recently reported type strain *E. glycinis* CCBAU from root nodules of *G. max* in China. A single strain TL4 clustered with *E. adhaerens* LMG 20216^T and *E. sesbaniae* CCBAU 65729^T (isolated from *Sesbania cannabina*, China). Microsymbionts of legume, *Trigonella foenum-graecum* (TFG22 and TFG64) as expected, shared close similarity with type strain *E. medicae* A321^T (*Medicago truncatula*, France). Few mimosoid strains formed distinct lineages within genus *Ensifer* (Fig. 2.1).

The genera in the subfamily Caesalpinioideae found in arid and semiarid regions of India are non-nodulating including species of *Cassia*, *Senna*, and *Parkinsonia* except the genus *Chamaecrista*. *Chamaecrista pumila* is a basal legume in which microsymbionts remain trapped in infection thread and are not dropped out, and these infection threads with symbiosomes are called as fixation threads. Novel findings were made related to microsymbionts of *C. pumila* in the Thar Desert. The genus *Chamaecrista* is known to be nodulated by slow-growing nitrogen-fixing *Bradyrhizobium* strains all over the world (Beukes et al. 2016; dos Santos et al. 2017). However, *C. pumila* growing in arid and semiarid regions of the Thar Desert of India is nodulated by fast-growing *Ensifer* in addition to slow-growing *Bradyrhizobium*. This is probably the first report of *Ensifer* nodulating *C. pumila*. This suggests that the host legume and naturalized fast-growing *Ensifer* of Indian Thar Desert have coevolved and both gained the ability to interact and form a symbiotic association (Rathi et al. 2018).

The limitation of 16S rRNA gene phylogeny is that closely related species cannot always be distinguished due to high level of sequence conservation. Therefore to determine the exact taxonomic position of these *Ensifer* strains, multilocus sequence analysis (MLSA) was performed for few selective strains. The MLSA based on conserved protein-coding housekeeping genes (*glnII*, *atpD*, *recA*, and *dnaK*) suggests that the Thar Desert *Ensifer* strains associated with root nodules of members of the papilionoid, mimosoid, and caesalpinioid legumes are novel species of *Ensifer* as they are significantly divergent from existing type strains of *Ensifer* (Tak et al. 2016b; Sankhla et al. 2017; Rathi et al. 2018). The *Tephrosia*-*Ensifer* (TP6 and TW10) strains were characterized at genomic level and designated as novel species of *E. aridi* (Le Queré et al. 2017). These Thar Desert *Ensifer* strains were found to be genetically identical to strains isolated from other parts of the world such as *Ensifer* strains from root nodules of *Acacia raddiana* and *Acacia*

gummifera from Merzouga Desert in Morocco (Africa) and from wild species of *Phaseolus* (*Phaseolus filiformis*) in the hot desert of Baja California in Mexico. The core genomes of six *Ensifer* strains from three different continents (Asia, Africa, and America) were compared with *Ensifer* type strains. Genome-based species delineation tools such as average nucleotide identity (ANI) and in silico-based DNA-DNA hybridization (DDH) demonstrated that they belong to a new species of *Ensifer*; however these strains were symbiotically distinct (*sym* genes continent specific). The genomic data suggested several conserved genes specific to genus *Ensifer* (Le Queré et al. 2017). Previously published work and the finding of predominance of *Ensifer* in hot-arid alkaline soils of Thar Desert suggest that *Ensifer* species have been mostly isolated from legumes growing in the arid and alkaline soils of Old World and New World (Tak et al. 2016b; Shamseldin et al. 2017; Sankhla et al. 2017; Rathi et al. 2018). Exception to this is *E. medicae* that nodulates species of *Medicago* in acidic soils (Garau et al. 2005). These findings are supported by the comparative genomic studies on *Ensifer* microsymbionts nodulating soybean growing in alkaline soil of China, suggesting that the strains of *Ensifer* have specific genes for adaptation to alkalinity, low water potential, salt stress, and high temperature (Tian et al. 2012).

Our studies suggest that the *Ensifer* strains associated with mimosoid members (*Vachellia*, *Senegalia*, and *Mimosa*) of the Thar Desert on the basis of their symbiotic genes are closer to type strain *E. arboris* (isolated from *Prosopis chilensis*, Sudan) [Fig. 2.2]. In species phylogeny these strains shared close similarity with *E. saheli*; this incongruence with symbiotic gene phylogeny is due to horizontal transfer of the *sym* genes (Gehlot et al. 2013; Sankhla et al. 2017; Choudhary *et al*. 2017, 2018). *Ensifer* strains affiliated with papilionoid and caesalpinioid legumes in the Thar desert showed maximum similarity to symbiotic genes (*nodA* and *nifH*) of closely related type strains (*E. fredii*, *E. glycinis*, *E. shofinae*, *E. sojae*, and *E. xinjiangensis*) isolated from root nodules of *G. max* and with broad host range strain (*Ensifer* sp. NGR234) (Tak et al. 2016a, b; Sankhla et al. 2018; Rathi et al. 2018) (Fig. 2.2). This phylogenetic incongruence observed in species and symbiotic gene phylogeny of Thar Desert *Ensifer* strains may be attributed to soil alkalinity and hot-arid conditions that play a major role in the evolution of these rhizobial strains. In contrast to this, the symbiotic essential genes of TFG strains had intermediate sequences diversified from closely related *E. meliloti* and *E. medicae*, as observed in their species phylogeny based on 16S rRNA gene (Gaur et al. 2018) [Fig. 2.2]. It was surprising to note that no *E. aridi* type of strains was isolated from root nodules of various mimosoids (*Vachellia*, *Senegalia*, and *Mimosa*) studied from the Thar Desert, although *E. aridi* (African strains LMR001 and LMR13) have been recovered from the species of *Acacia* (*Vachellia*) from Morocco, Africa (Fig. 2.2). The *sym* gene phylogenies of different *E. aridi* strains from three continents (American strain LEM457) clearly indicate that these strains are genetically identical but harbor different symbiotic genes specific to local environmental conditions (Fig. 2.2). The Indian *E. aridi* strains harbor novel *sym* genes diversified from *E. fredii* and cross-nodulating papilionoid, mimosoid, and caesalpinioid wild legumes as well as crops (Tak et al. 2016b; Le Queré et al. 2017).

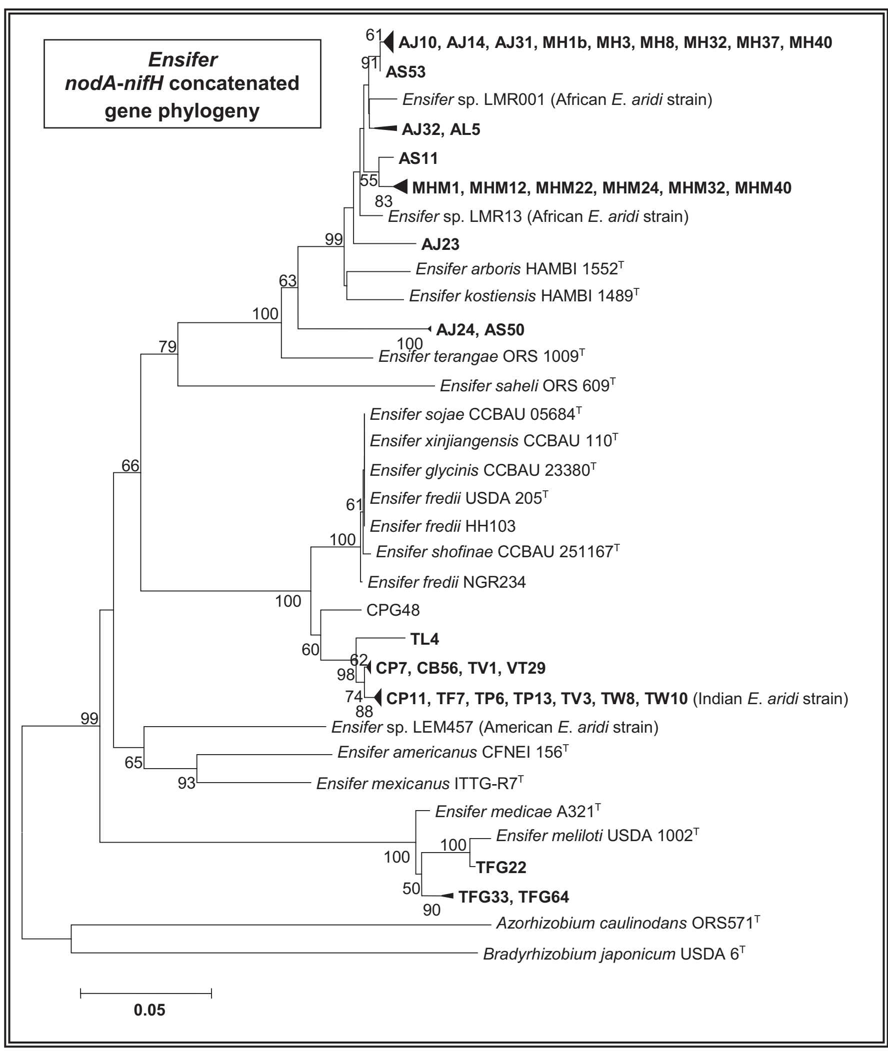

Fig. 2.2 Maximum likelihood concatenated phylogenetic tree of **Thar Desert *Ensifer*** strains isolated from root nodules of various wild legumes of Western Rajasthan based on ***nodA-nifH*** concatenated gene sequences. Bootstrap values more than 50% calculated for 1000 replications are indicated at internodes. The scale bar indicates 5% nucleotide substitution per site. (Abbreviation: superscript T stands for type strain)

2.3.2 Phylogenetic Diversity of Thar Desert *Bradyrhizobium* Strains

Among the various wild legumes of the Thar Desert studied, the slow-growing bradyrhizobial strains were also recovered from root nodules of species of *Tephrosia* (tribe Millettieae, Papilionoideae) (Gehlot et al. 2012; Ojha et al. 2015), *Crotalaria*

burhia (tribe Crotalarieae, Papilionoideae) (Ojha et al. 2015; Sankhla et al. 2018), *Alysicarpus vaginalis* (tribe Desmodieae, Papilionoideae) (Rathi et al. 2017), and *Chamaecrista pumila* (tribe Cassieae, Caesalpinioideae) (Ojha et al. 2015; Rathi et al. 2018). The slow-growing *Bradyrhizobium* strains isolated from root nodules of these wild herbs and under shrubs on the basis of 16S rRNA and *sym* (*nodA* and *nifH*) gene phylogeny shared close affinity with the type strain *B. yuanmingense* which was initially isolated from wild legume (*Lespedeza cuneata*) of tribe Desmodieae in China (Yao et al. 2002) (Figs. 2.3 and 2.4). From India, strains sharing similarities with type strains *B. yuanmingense*, *B. liaoningense*, *B. elkanii*, and *B. japonicum* have been isolated from *G. max* growing in Madhya Pradesh, Uttar Pradesh, and Tamil Nadu having hot-arid climate and being mostly neutral to alkaline soils (Appunu et al. 2008, 2009b; Vinuesa et al. 2008). The *B. yuanmingense* has also been reported to nodulate *V. mungo*, *V. radiata*, and *V. unguiculata* growing in regions of Uttar Pradesh, Andhra Pradesh, and Tamil Nadu (Appunu et al. 2009a) and in another crop *Macrotyloma uniflorum* growing in two agro-eco-climatic regions of South India (Appunu et al. 2011). As per previous studies from various parts of India, *B. yuanmingense* is often the preferred symbionts of crops (*G. max* and *Vigna* spp.) in the hot-dry tropical climate and alkaline soils. The existing geographical factors and alkaline soil in the Thar Desert are playing a role in the dominance of *Ensifer* and the sporadic occurrence of *B. yuanmingense*-like strains in root nodules of species of *Tephrosia*, *Alysicarpus*, and *Chamaecrista* in addition to strains of *Ensifer* which might be the preferred/primary rhizobia compared to other rhizobial genera.

In contrast to the Thar Desert bradyrhizobial strains (from mega clade-I), novel species of *Bradyrhizobium* belonging to both mega clade-I and clade-II have been recently isolated from little-studied legumes *Eriosema chinense* (tribe Phaseoleae, Papilionoideae) and *Flemingia vestita* (tribe Phaseoleae, Papilionoideae) growing in acidic soil of the sub-Himalayan region of the Indian state of Meghalaya (Ojha et al. 2017). These strains isolated from acidic soils of Shillong harbor novel *nodA* and *nifH* genes (Fig. 2.4). This suggests that although selection of rhizobia by the host plants depends upon molecular signaling between the two partners, that too is influenced by ecological factors such as soil alkalinity, soil acidity, precipitation, and soil nutrients' availability in the region (Rathi et al. 2018). Caesalpinioid legume *C. pumila* in Thar Desert was nodulated by novel strains of *Ensifer* and strains divergent from *B. yuanmingense*, whereas in acidic soils and wet soils of northeastern state Meghalaya, it was found to be exclusively nodulated by diverse species of *Bradyrhizobium* belonging to both mega clade-I and clade-II. Significant symbiotic (*nodA* and *nifH* gene) diversity was observed in *C. pumila* strains isolated from acidic soils of Shillong (CPS) (Fig. 2.4). In the mimosoid legumes of Thar Desert such as *V. leucophloea* (Choudhary et al. 2017), *P. cineraria*, and *Dichrostachys cinerea* (Unpublished data, HS Gehlot), the primary dominant root nodule microsymbionts were fast-growing species of *Ensifer*, and occasionally slow-growing *Bradyrhizobium* strains close to *B. yuanmingense* were also reported.

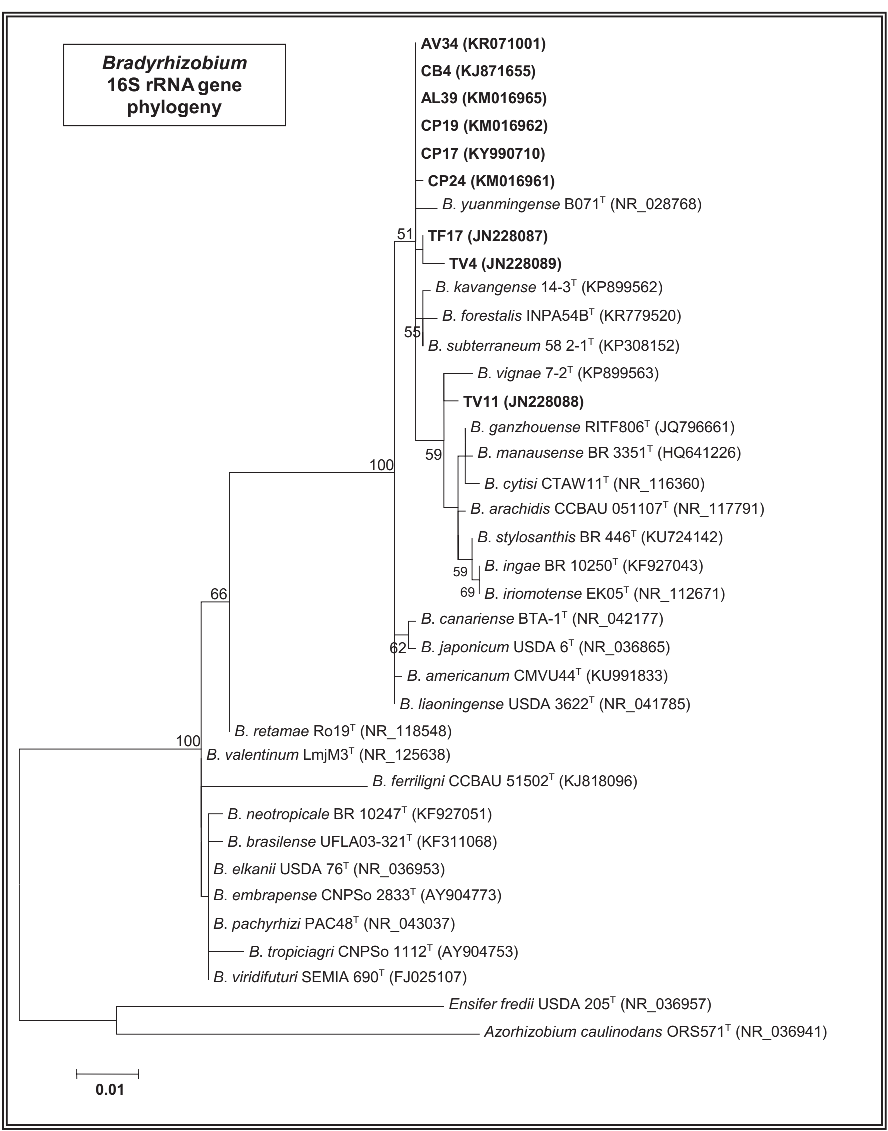

Fig. 2.3 Maximum likelihood phylogenetic tree of **Thar Desert *Bradyrhizobium*** strains isolated from root nodules of various wild legumes of Western Rajasthan based on **16S rRNA** gene sequences. Bootstrap values more than 50% calculated for 1000 replications are indicated at internodes. The scale bar indicates 1% nucleotide substitution per site. (Abbreviation *B.* stands for *Bradyrhizobium* and superscript T stands for type strain)

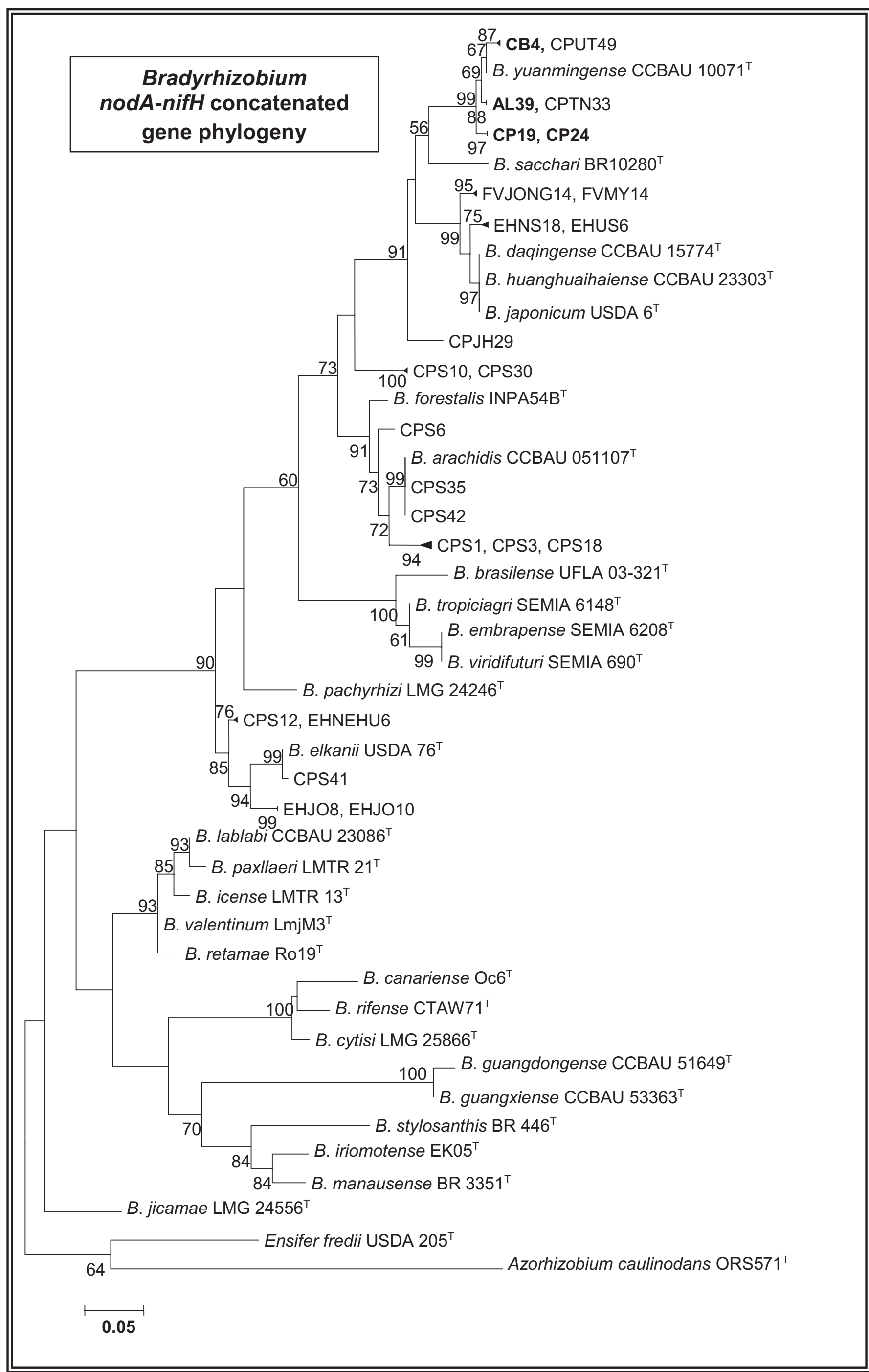

Fig. 2.4 Maximum likelihood concatenated phylogenetic tree of **Thar Desert *Bradyrhizobium*** strains isolated from root nodules of various wild legumes of Western Rajasthan compared

2.4 Conclusions and Future Perspectives

Since the beginning of exploration of native and wild legumes from various geographical regions all over the world, the number of rhizobial genera and species has increased tremendously due to which rhizobial classification is changing. The soil alkalinity, low precipitation, and high temperature in the arid and semiarid regions of the Thar Desert are responsible for evolution and diversification of novel *Ensifer* strains when compared to slow-growing *Bradyrhizobium* from tropical and subtropical acidic soil all over the world including temperate regions. Novel *Ensifer* strains are dominant microsymbionts associated with legumes in the region. Majority of the *Ensifer* strains adapted to arid conditions of the Thar Desert are promiscuous in nature, showing geographical clustering and mosaic pattern in nucleotide sequences of housekeeping as well as symbiotic genes. Hostile environmental conditions are an important factor underlying horizontal gene transfer among related and diverse genera and species of bacteria. Molecular phylogeny of Thar Desert rhizobia provided information about their evolution and diversification and helped in better understanding the biogeography of rhizobial strains in India and in global context. Genomic characterization of diverse nitrogen-fixing novel strains from different climatic conditions will enrich our knowledge about the unique features of these strains which may open new fields for biotechnologists to improve existing microsymbiont associated with legume crops. Such studies are essential for strengthening the microbial resources in terms of agriculturally important microbes and can reduce the use of chemical fertilizers that are causing environmental pollution and adversely affecting the economy of farmers.

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Fig. 2.4 (continued) with *Bradyrhizobium* strains isolated from other sampling sites in India having neutral [Tamil Nadu (TN), Jharkhand (JH), and Uttarakhand (UT)] and acidic [Shillong, Meghalaya] soils, based on ***nodA-nifH*** concatenated gene sequences. Bootstrap values more than 50% calculated for 1000 replications are indicated at internodes. The scale bar indicates 5% nucleotide substitution per site. (Abbreviation *B*. stands for *Bradyrhizobium* and superscript T stands for type strain)

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Soil Microbiota and Sustainable *Jhum* Agroecosystem

3

S. R. Joshi, Donald Bareh, and Aishiki Banerjee

Abstract

The exclusivity of the northeastern region of India lies in its diversity in terms of its biological aspects and also the array of different practices that the region still continues to harbor in their own indigenous ways. The well-knitted practices with diverse cultures span across the changing altitudes of the sisterly states of the northeast. Such environments provide a wide opportunity for the documentation and bioprospection of biotechnologically useful microbes as most of the microbial diversity here remains unknown. The agricultural land in this region is mostly subjected to the shifting type of agriculture which is commonly known as *jhum* farming. Management of the shifting practice in agronomy depends on the period the land is cultivated and the stretch that is fallowed. These factors greatly impact on whether or not the shifting cultivation system as a whole suffers a net loss of nutrients over time. Recently, extensive studies have been focused on soil microbial diversity of these shifting cultivated lands to keep at par with the enormous amount of effort that is being made worldwide by microbial ecologists to identify microorganisms in various type of environmental samples. As the field of *jhum* offers an environment that undergoes a tilt and shift in its nutrients along with other components, a competitive environment for the micro-dwellers of the soil is created. Hence, studying the microbial profile mostly related to the area of plant growth promotion (PGP) from differently aged fallows of *jhum* can provide a clear picture of the underlying ecosystem that influences the aboveground activities with an opportunity to design bioinoculants consortium for eco-restoration of *jhum* fields.

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Bioprospecting · Fallows · *Jhum* · Microbial diversity · Soil biota · Shifting agriculture · Plant growth promotion (PGP)

3.1 A Light on the Shifting Cultivation Practices in Northeast India

Shifting cultivation, one of the most debatable forms of farming practice in current times, has a noteworthy feature of universality in the tradition of agricultural practice of slashing and burning the forest and thereby using the cleared land for cropping. This type of farming system, often been referred to as "slash-and-burn" or "swidden" type of agriculture, combines both cropping and fallow periods in its land rotation (Nye and Greenland 1960; Kunstadter et al. 1978; Ramakrishnan and Toky 1981). The practice of shifting cultivation in the tropical parts of Asia slightly varies from the other parts of the world as this practice is concentrated on plains of alluvial soils or hilly terrains with rice cultivation. The cycle of the shifting cultivation involves several steps of which the characteristics being the rotation of fields rather than rotation of crops, starting with the selection of plots, slashing and cleaning of the natural standing vegetation, and burning, followed by a short cropping period, harvesting, and then leaving fallow when the land shows signs of exhaustion in productivity (Fig. 3.1).

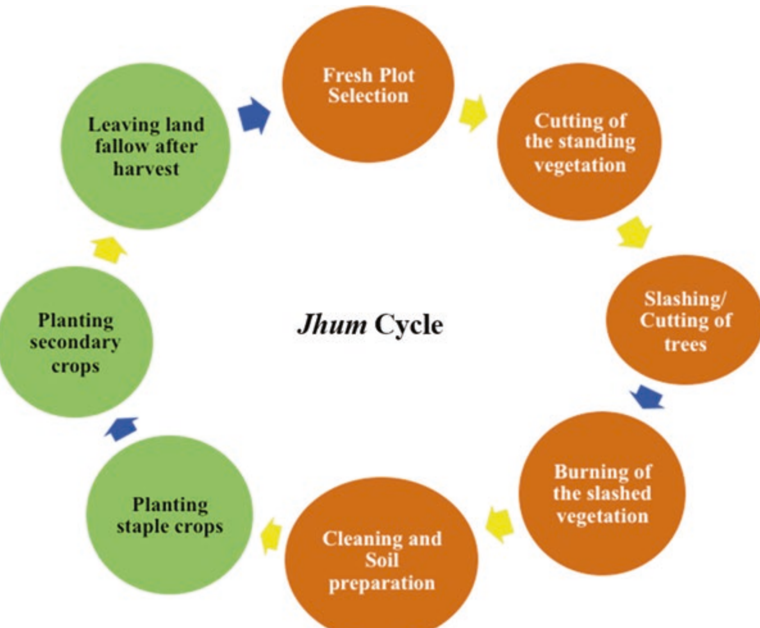

Fig. 3.1 The cycle of shifting (slash-and-burn) cultivation

Shifting cultivation remains the main source of employment for large sections of the rural people who depend on agriculture for their livelihood and occupation in various parts of the Indian subcontinent concentrated in the states of Assam, Mizoram, Meghalaya, Manipur, Nagaland, and Arunachal Pradesh and also in some belts in Orissa, Madhya Pradesh, Andhra Pradesh, and Kerala (Jha 1997; Freeman 1999). In different states it is known by different names – mostly known as *jhum* in the northeastern states of Assam, Nagaland, Mizoram, and Meghalaya, *punamkrishi* in Kerala, *podu* in Andhra Pradesh and Orissa, and *bewara*, *marhan*, *penda*, and *beera* in different parts of Madhya Pradesh (Eden 1987). In the Indian subcontinent, over 86% of the people living in the hills of the northeast are dependent on *jhum* cultivation (Patel et al. 2013). About 20 lakhs ha of forests are cleared every year by felling and burning the trees and shrubs (Satapathy et al. 2003). In 1980, about 1,326,000 ha were under *jhumming* which increased to 1,685,000 ha in 1990. Out of 4.0 Mha of net sown area, about1.6 Mha area is under *jhumming* in this region (Kumar et al. 2008) (Figs. 3.2 and 3.3).

3.1.1 Microbial Diversity Pertaining to Shifting Cultivation

Microorganisms are the ubiquitous custodian of the Earth that have been found to exist in almost all climatic conditions in soil, air, or water, including those once considered to be most unlikely to support life like that in the Arctic and the Antarctic, natural hot springs and oceanic hot vents, and deep within the rocks (Colwell 1997). For more than 3 billion years, microbes have been existing on this planet, and their competencies to modify to several environments make them prominent organisms. Natural microbial diversity comprises of an enormous range of microorganisms that exert a strong influence on biogeochemical cycles of carbon, nitrogen, and sulfur (Balvanera et al. 2006; Golubiewski 2010). Understanding of microbial diversity and its uses is scarce in spite of their immense importance in human welfare. The

Fig. 3.2 The burning and fallowing activities in a shifting cultivation field

Fig. 3.3 A typical shifting cultivation area in Nagaland

problem of how to preserve microorganisms and their gene pools persists as the enormity of microbial diversity becomes apparent and asserts itself ever more forcibly. The Earth manifests an estimated number of 1,000,000 bacterial and 1,500,000 fungal species, yet fewer than 4,000 and 70,000 have been designated in case of bacteria and fungi, respectively (Heywood and Watson 1995). Although there has been a swift rise in the strategies recommended for conservation of biodiversity, none seem to consider microorganisms (Bhattacharjee et al. 2012). The soil has been an exceptional culture medium for growth and maintenance of a wide-ranging microbiota. Although it appears inert and static, it is considered as a medium pulsating with life. The soil microorganisms are key machinery that determine life support functions playing important roles in biogeochemical cycles (van Elsas et al. 2012). "Microbes orchestrate life on earth" has been a journey toward understanding, managing, and protecting microbial ecosystems but mostly concentrating on philosophical than methodical aspects (Kowalchuk et al. 2008). Only in the recent past, global climate change has been an important criterion of the social agenda owing to the growing realization that the Earth's resources are being used in an unsustainable fashion.

With increasing population density, agricultural productivity needs to be increased, and with it farmers will depend on extensive use of various chemical fertilizers (Wartiainen et al. 2008; Adesemoye et al. 2009) as these have become the main source for providing nitrogen, phosphorus, and potassium (Shenoy et al. 2001). Inadvertently, these chemicals have caused a negative impact on the environment, some being recalcitrant and contaminants of the environment (Adesemoye et al. 2009). One of the ways to cut down the use of these fertilizers is the utilization of the naturally occurring plant growth-promoting rhizobacteria (PGPR) in the soil. Moreover, the use of efficient and potential inoculants can be considered an important strategy for ecological management and for reducing environmental hazards by decreasing the use of chemical fertilizers (Alves et al. 2003; Balasubramanian et al. 2004; Hungria et al. 2010, 2013). Bacteria have different parameters or properties that influence plant growth and hence play a dynamic role in the sustainability of

Fig. 3.4 A pictorial depiction of the *jhum* cycle showing the different land transitions

agriculture through assimilated nutrient supply (Rengel and Marschner 2005). To achieve maximum benefits in terms of fertilizer savings and better growth, the PGPB-based inoculation technology is proposed along with appropriate levels of fertilization (Shenoy et al. 2001; Adesemoye et al. 2009). However, it is important to lay major emphasis on the application of efficient microbial inoculants in terms of monoculture inoculants or consortium for better crop productivity (Fig. 3.4).

Native microbial communities in particular from the shifting cultivated areas in the Indian subcontinent are less explored as compared to the study of microbial communities from other agricultural fields. While microorganisms are ubiquitous in nature, their distribution is largely governed by environmental specificities. Bacteria are typically the first organisms to sense and respond to chemical and physical changes in their environment (Burns et al. 2013). Microbial populations and their responses to stresses have been traditionally studied at the process level, in terms of total numbers of microorganisms, biomass, respiration rates, and enzyme activities, with little attention being paid to responses at the community or the organismal levels. Microbial communities and their processes need to be examined in relation

to not only the individuals that comprise the community but the effect of perturbations or environmental stresses on those communities (Joshi et al. 1991; Desai et al. 2009). The study of bacterial diversity is therefore crucial for understanding the dynamics of many ecological processes.

Microorganisms are key to soil health, as they form an essential correlation between the availability of soil nutrients and crop productivity. They perform basic functions in soil formation and in biogeochemical cycling of nutrients aiding the transformation of organic and inorganic nutrients. Fungi are an important group of microorganisms that can survive diverse soil conditions, owing to their ability to be able to decompose and break down a wide range of organic matter by the production of extracellular enzymes. This helps fungi to adapt to adverse environments while maintaining the balance of essential nutrients like carbon, in soil (Žif˘cáková et al. 2016). They grow in every kind of environment and can tolerate varying ranges of soil pH and temperature conditions (Frac et al. 2015). The biodiversity of soil fungi is determined by the diversity and composition of the thriving floral and bacterial community. Also, fungi are governed by a number of abiotic factors such as temperature, soil pH, and salinity, which have a profound effect both on the diversity and their activity in the soil (López-Bucio et al. 2015; Rouphael et al. 2015). Based on their functions, soil fungi can be divided into mainly three groups – biological controllers, ecosystem regulators, and organic matter decomposers (Swift 2005; Gardi and Jeffery 2009).

Several bacteria and fungi fall into the category of promising growth promoters, and a number of them have been studied for their phosphate-solubilizing and antagonistic properties with respect to potential use for plant growth and biocontrol (Tilak et al. 2005; Lugtenberg and Kamilova 2009; Islam et al. 2016; Banerjee et al. 2017). In view of the ecological specificity of such organisms, isolation and characterization of native microorganisms from diverse ecological niches has become particularly essential (Wang et al. 2007) (Table 3.1).

3.2 Microbes and Their Role in Plant Growth Promotion

The microbial community dominant at any point of time at a particular place differs greatly due to different plant species and their interactions with the soil microbiome (Marschner et al. 2001; Kourtev et al. 2002). In both cultivated and natural environments, soil microbes play a crucial role in improving soil health by promoting plant growth. Bacteria colonizing the rhizosphere and root region in soil that boost plant growth are referred to as plant growth-promoting rhizobacteria (PGPR) (Davison 1988; Lemanceau 1992; Kloepper 1994). The root exudates released in the rhizosphere act as chemical signals for a diverse community of microorganisms, and the resulting microbial community known as the rhizomicrobiome (Hinsinger et al. 2009; Chaparro et al. 2013) allows microorganisms to thrive and flourish, making the soil rich and fertile (Walker et al. 2003). This group of PGPR includes different bacterial species belonging to *Azotobacter*, *Arthrobacter*, *Azospirillum*, *Acetobacter*, *Alcaligenes*, *Acinetobacter*, *Erwinia*, *Enterobacter*, *Herbaspirillum*, *Pseudomonas*,

Table 3.1 Bacteria and fungi reported from shifting cultivated fields of Northeast India

Bacteria	**Sampling site**	**Sampled from**	**Microorganisms involved**	**References**
	Lobi of Papumpare District, Itanagar, Arunachal Pradesh	Fallowed and fired plots under shifting cultivated lands	*Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus clausii*, *Bacillus megaterium*, *Bacillus* sp., *Pseudomonas stutzeri*, *Pseudomonasa eruginosa*, *Bacillus thuringiensis*	Pandey et al. (2011)
	Jhum sites of Northeast India	Fallow soil	*Curtobacterium oceanosedimentum*, *Bacillus methylotrophicus*, *and Bacillus cereu*s plant growth promoters	Banerjee et al. (2017)
	Varying fallow periods across Mizoram	Dry forest litter falls of *jhum* fallows	Cellulose Degrading Microorganisms	Sangma and Thakuria (2018)
Fungi	Arunachal Pradesh	Soil of *jhum* sites	*Trichoderma*, *Penicillium*, *Paecilomyces*, *Cladosporium*, *Aspergillus*, *Rhizoctonia*, and *Coniella*	Jain et al. (2016)
	Jhum Fallows	Weedsinfesting *jhum* fields *Spilanthespaniculata* and *Ageratumconyzoides*	Group of *Deuteromycetes* like *Acrimonium*, *Arthroderma*, *Aspergillus*, *Botrytis*, *Cercospora*, *Chalaropsis*, *Chrysosporium*, *Cladosporium*, *Fusarium*, *Geotrichum*, *Humicola*, *Metarhizium*, *Monilia*, *Mortierella*, *Mucor*, *Nectria*, *Nigrospora*, *Penicillium*, *Staphylotrichum*, *Trichoderma, and Verticillium*	Majumder et al. (2008)
	Jhum sites of Northeast India	Fallow soil	*Penicillium virgatum*, *Metarhizium pinghaense*, and *Penicillium stratisporum*	Banerjee et al. (2017)

Burkholderia, *Serratia*, and *Bacillus* (Glick 1995; Probanza et al. 1996; Rodríguez and Fraga 1999). The beneficial plant-microbe interactions in the rhizosphere are the factors behind plant health and soil fertility, and these microorganisms are very important in the biogeochemical cycles of both organic and inorganic nutrients and maintenance of soil health and quality (Kloepper and Schroth 1978; Jeffries et al.

2003). These microbes improve plant growth through traits such as phosphate solubilization, auxin synthesis, and siderophore production (Sharma et al. 2014). The interactions in the rhizosphere play a pivotal role in transformation, mobilization, and solubilization of nutrients from a limited pool in the soil to uptake by the crop plants.

Fungi can control the growth of pathogenic microorganisms and pest infestations like mycorrhizal fungi which protect plants against pathogens and also help in increased nutrient uptake. They regulate many physiological processes in soil and also play a role in the formation of soil structure (Bagyaraj and Ashwin 2017). They also help in the stabilization of soil organic matter by decomposition of residues (Treseder and Lennon 2015). With higher complexity and diversity of soil fungi, the rate of decomposition of organic matter also increases helping better absorption of nutrients by the plants and aiding nutrient cycling; the plant, in turn, provides photosynthetic carbon to the soil fungi for its growth, thus increasing their diversity through the energy resources obtained (Hiscox et al. 2015; Sláviková et al. 2002; Ponge 2013). Fungi are also involved in various other processes such as hormone production, nitrogen fixation, and tolerance of plants to drought conditions (Jayne and Quigley 2014; Baum et al. 2015; El-Komy et al. 2015). Certain fungal species act as good bioaccumulators as they are able to efficiently absorb toxic metals like lead, cadmium, copper, etc. in their fruiting bodies (Baldrian 2003).

The use of PGPR is steadily increasing in agriculture and offers an attractive way to replace chemical fertilizers and pesticides (Dey et al. 2004; Herman et al. 2008; Minorsky 2008). The utilization of bio-fertilizers having beneficial microorganisms as an alternative to synthetic chemicals is known to develop plant growth through supplementing plant nutrients and may help to sustain soil health and productivity (O'Connell 1992). Lately, there has been a growing interest in the research areas encompassing environment-friendly, sustainable, and organic agricultural or horticultural practices (Karlidag et al. 2007).

3.2.1 Impacts and Benefits of PGPR

PGPRs can employ a positive impact on plant growth in two different ways: directly or indirectly. The direct mechanisms of growth promotion generally entail different pathways of the bacterium, providing the plant with a compound that directly benefits the plant in contact or facilitates the plant in the uptake of certain nutrients (Glick 1999). It includes the synthesis of phytohormones like IAA and GAA (Xie et al. 1996), N2 fixation (Weniger 1992), solubilization of inorganic P and mineralization of organic phosphate, making P available to plants (Subba-Rao 1982; Rodríguez and Fraga 1999), reduction of membrane potential of the roots (Bashan and Levanony 1991), and synthesis of some enzymes that modulate level of plant hormones like ACC deaminase (Glick et al. 1998; Shah et al. 1998). On the other hand, the indirect enhancement of plant growth occurs when bacteria reduce or prevent some of the lethal effects of a phytopathogenic organism by one or more mechanisms. This occurs when bacteria synthesize antibiotics (Sivan and Chet

1992), produce induced systemic resistance against a number of plant diseases (Jetiyanon and Kloepper 2002) or produce iron-chelating compounds called siderophores (Leong 1986; Gray and Smith 2005). Rhizosphere bacteria multiply to high densities on plant root surfaces where root exudates and root cell lysates provide ample nutrients. Sometimes, they exceed 100 times those densities found in the bulk soil (Campbell and Greaves 1990).

3.2.2 Role of PGPRs in Shifting Cultivated Fields

Shifting cultivation or *jhum* has its own biological merits and demerits. The uncultivated phases help in regeneration and enrichment of different soil properties along with an increase in soil microbial biomass and hence its diversity (Pandey et al. 2010). The survival of the plant growth promoters under such circumstances and their regeneration in *jhum* fallows is important for the health of cultivated crops. *Jhum* soils are usually characterized by depleting nutrients, like reduction of inorganic carbon and depletion in nitrogen content (Ramakrishnan and Toky 1981). There have been reports of a decrease in soil enzymes such as soil phosphatase activity and β-glucosidase activity after burning of forests (Boerner et al. 2000). In context to such depleting soil conditions, it has become necessary to study the presence of plant growth-promoting bacteria present in these soils and their role to improve the different aspects of soil conditions which in turn will improve the plant growth, making the system a sustainable one (Table 3.2).

3.3 Soil Fungi in the Context of *Jhum* Cultivation

In the *jhum* system of cultivation, fire is an important tool used as a means to convert standing forest covers to agricultural lands. This practice improves soil fertility as there is a sudden increase in the availability of nutrients due to heat while reducing the microbial population in soil (Deka and Mishra 1983). However, fungi seem to be affected the least among the group as they have a higher capacity to proliferate owing to their hyphal structures or the mycelia (Whipps 2001). A study carried out in shifting cultivated field of Arunachal Pradesh reveals the presence of soil fungi, mainly belonging to the genus of *Trichoderma*, *Penicillium*, *Paecilomyces*, *Cladosporium*, *Aspergillus*, *Rhizoctonia*, and *Coniella*. These were those that were able to survive the fire operation as the samples for isolation were collected just after burning (Jain et al. 2016). The study on the population of fungi in *jhum* fields amended by organic fertilizers was carried out by Swer et al. (2011) which revealed the presence of at least 120 fungal isolates belonging to the groups of *Ascomycotina*, *Deuteromycotina*, and *Zygomycotina*. Of these, 24 species of *Penicillium*; 8 species of *Aspergillus*; 4 species each of *Paecilomyces*, *Verticillium*, *Talaromyces*, and *Trichoderma*; and 6 species each of *Fusarium*, *Mortierella*, *Mucor*, and *Acremonium* were obtained. It was concluded from this study that the addition of organic fertilizers increased the population and survivability of soil

Table 3.2 PGPRs along with associated properties obtained from Northeast India

Sampling site	Sampled from	PGPRs	Properties	References
Jorhat, Assam	Soil	*Bacillus cereus* and *Pseudomonas rhodesiae*	Enhanced crop production	Kalita et al. (2015)
Manipur	Tomato rhizosphere	*Streptomyces virginiae* *Streptomyces tanashiensis* *Streptomyces polychromogenes* *Streptomyces pratensis* *Streptomyces tunisiensis* *Streptomyces ederensis* *Streptomyces vinaceus* *Streptomyces cinereoruber*	Phytostimulating and biocontrol agents	Kshetri et al. (2018)
Imphal, Manipur	Rhizosphere soil from French bean field	*Burkholderia*, *Cepacia*	Biocontrol agent	Devi et al. (2012b)
Meghalaya	Endophytes of *Centellaasiatica*	*Serratia marcescens*, *Bacillus subtilis*, *Serratia* sp.	Showing antifungal activity	Nongkhlaw and Joshi (2014)
Northeast India	Shifting cultivated fields	*Bacillus methylotrophicus*	Enhancement of rice growth	Banerjee et al. (2017)
Darjeeling tea estates	Tea soil rhizosphere	*Yokenella regensburgei*, *Brevibacillus agri*, *Klebsiella michiganensis*, *Aneurinibacillus aneurinilyticus*, *Sporosarcina koreensis*, *Pseudomonas monteilii*, *Burkholderia territorii*, *Burkholderia territorii*	Plant growth-promoting properties like siderophore production and IAA production	Dutta and Thakur (2017)
Meghalaya	Soil sample	*Bacillus cereus*, *Bacillus subtilis*	Thermostable α-amylase producers	Devi et al. (2010)
Meghalaya	Sacred grove soil	*Bacillus*, *Paenibacillus*, *Lysinibacillus*, and *Viridibacillus*	Bioprospecting and plant growth-promoting *Bacilli*	Lyngwi et al. (2016)
Domiasiat, Meghalaya	Subsurface soils of uranium ore deposits	*Serratia* sp.	Can tolerate uranium along with PGP properties	Kumar et al. (2011)
Meghalaya	contaminated soil	*Staphylococcus equorum*, *Enterobacter* sp., *Bacillus subtilis*	Insecticide (endosulfan) degrader applicable in fields	Bhattacharjee et al. (2014)

fungi and aided in their spore formation (Swer et al. 2011). The study in *jhum* cultivated fields on the decomposition of weeds, namely, *Spilanthes paniculata* and *Ageratum conyzoides* L., suggests fungal diversity to be dependent on the decomposition period and nutrient concentration of the weeds. The majority of the fungal isolates that were isolated belonged to the group of Deuteromycetes. The diversity of fungi was found to be highest in the foliage of *Ageratum conyzoides*, followed by root portion of *Spilanthes paniculata* and *Ageratum conyzoides.* The major genera of soil fungi that were obtained from this study were mainly *Acremonium*, *Arthroderma*, *Aspergillus*, *Botrytis*, *Cercospora*, *Chalaropsis*, *Chrysosporium*, *Cladosporium*, *Fusarium*, *Geotrichum*, *Humicola*, *Metarhizium*, *Monilia*, *Mortierella*, *Mucor*, *Nectria*, *Nigrospora*, *Penicillium*, *Staphylotrichum*, *Trichoderma*, *and Verticillium* (Majumder et al. 2008). The assessment of the microbial diversity of the vegetational zones along the Eastern Himalayan region revealed a diverse group of microfungi thriving in the particular belt. The isolates obtained belonged to at least 59 different genera of soil fungi among which *Oidiodendro*, *Cladosporium*, *Aspergillus*, *Acremonium*, *Penicillium*, and *Humicola* were noted to be dominant. The most commonly found genera in agricultural soils were *Penicillium*, *Humicola*, and *Acremonium* (Sharma et al. 2015). The most dominant genus of fungal species found in this study was *Oidiodendron*, which are known to produce various enzymes enabling them to act as decomposers of a different variety of substrates in soil (Rice and Currah 2005). The physicochemical properties of soil, pH, temperature, type of vegetation found, and altitude were seen to be factors governing the soil fungal diversity (Pandey et al. 2006; Devi et al. 2012a).

3.3.1 Mycorrhizal Fungi in *Jhum* Cultivation

Certain types of fungi, which thrive in the rhizosphere of plants, are able to influence plant growth as they are involved in direct physical interaction with the plants; they are known as mycorrhizal fungi. They are an important group of beneficial fungi which are most widely used in agriculture and studies suggest that their application is known to significantly enhance crop productivity (Bagyaraj and Ashwin 2017; Smith and Read 2008; Thilagar and Bagyaraj 2015). Mycorrhizal fungi form symbiotic associations with roots of plants and help increased root structures, survival of plants in drought by improving water retention capacity of the soil, increase absorption of nutrients by plants and also in nutrient cycling (Azcón-Aguilar and Barea 1997). Some species of mycorrhiza like *Trichoderma* and *Glomus* have been reported to control diseases in crops owing to their antagonistic activity against pathogens (Dawidziuk et al. 2016); many strains of *Trichoderma* are used as biocontrol agents in horticultural plants (López-Bucio et al. 2015). The application of PGPRs and mycorrhizal fungi as a consortium is also seen to have a positive synergistic effect on the biodiversity of soil microbes and plant growth in horticulture (Azcón-Aguilar and Barea 1997;

De Coninck et al. 2015). The effect of such interactions can either be advantageous like the promotion of plant growth or sometimes could also be deleterious like causing diseases to the host plants (Voríškova 2013). In shifting or *jhum* cultivation, the soil is subjected to a number of disturbances especially owing to the fire used for clearing of vegetation along with other agricultural practices. Such disturbances bring about changes in the soil dynamics that affect the diversity and activity of mycorrhizal fungi thriving in the rhizosphere of plants leading to poor growth and decreased crop productivity (Bellgard 1994; Roldan et al. 1997). According to a study carried out on mycorrhizal fungi associated with *jhum* system, their population was found to be much lower in the fallow fields as compared to an adjacent undisturbed forest area, with at least 22.72% of total obtained species found to be absent in the fallows. The major genera of mycorrhizal fungi that were found in these sites were *Glomus*, *Acaulospora*, *Gigaspora*, *Entrophospora*, *Scutellospora*, and *Sclerocystis*. This loss could be due to the practice of slashing and burning which is characteristic of *jhum* cultivation (Singh et al. 2003). Also, it has been reported that disturbance and changes caused in the soil due to agricultural activities also leads to reducing the population of thriving mycorrhizal fungi (Miller 1979). Repeated cropping cycles in the same plot of land also render the soil infertile, and hence fields are left fallow and abandoned by the farmers (Duponnois et al. 2001). Mycorrhizal fungi were also found to dominantly colonize the roots of commonly found plant species, namely, *Crotalaria anagyroides*, *Eupatorium adenophorum*, and *Hedychium coronarium*, found in Meghalaya, with the major genus being *Glomus* and *Acaulospora.* The plant species were able to harbor a variety of mycorrhizal species of these genera (Songachan and Kayang 2011). *Solanum* species found in this region when investigated for mycorrhiza revealed the presence of at least four types of genus mainly *Glomus*, *Scutellospora*, *Gigaspora*, and *Acaulospora* (Songachan and Kayang 2012). A more in-depth study and investigation are required as to the diversity and distribution of mycorrhiza and the influence it has on the growth and composition of plant species growing in a particular niche.

3.4 Plant Growth-Promoting Rhizobacteria (PGPR) in *Jhum* Agriculture

Phosphorus is one of the most common elements on Earth yet remains unavailable to plants as most of it remains insoluble although abundant in both organic and inorganic forms (Goldstein 1986; Igual et al. 2001; Rodríguez et al. 2006). P is by far the most immobile and least available among other major nutrients like nitrogen making even most fertile soils P deficient. This is mainly due to the high reactivity of P with the inorganic compounds of calcium, iron, or aluminum present in the soil (Von Wandruszka 2006; Bianco and Defez 2010). Some species of bacteria such as *Pseudomonas*, *Bacillus*, *Rhizobium*, *Burkholderia*, *Achromobacter*, *Agrobacterium*,

Micrococcus, *Aerobacter*, *Flavobacterium*, and *Erwinia* have been reported to solubilize insoluble inorganic phosphate compounds such as tricalcium phosphate, dicalcium phosphate, hydroxyapatite, and rock phosphate in soil (Rodríguez and Fraga 1999; Khiari and Parent 2005). Phosphate solubilization has also been reported under environmentally stressed conditions under high levels of aluminum, iron, and desiccation (Vyas et al. 2007). The inoculation of phosphate solubilizers in nutrient-deficient soils can reduce the load of external application of chemical fertilizers (Gupta et al. 2015).

Another important aspect where bacteria impose plant growth promotion is by producing or regulating plant hormones that play a key role in its development and also to certain environmental responses (Glick 1995; Böttcher et al. 2010). Even though several auxins occurring in nature have been described, indole-3-acetic acid (IAA) is by far the most common and most studied auxin (Patten and Glick 1996; Bowen and Rovira 1999; Joo et al. 2005). IAA is reported to actively take part in promoting plant growth by plant cell proliferation, extension, and differentiation; initiating lateral and adventitious root development; stimulating seed and tuber germination; mediating responses to light, gravity, and florescence; and affecting photosynthesis, pigment formation, and biosynthesis of various metabolites resulting in elongation and vegetative growth (Glick 1995; Hardoim et al. 2008; Xie et al. 1996). It helps to build resistance to plant pathogens and improve survivability under stressful conditions, including biotic and abiotic stresses (Tsavkelova et al. 2006; Spaepen et al. 2007; Glick 2012). In general, bacterial IAA increases root surface area and length, thus providing the plant with a larger access to soil nutrients. The bacterial IAA, in turn, loosens the plant cell walls and as a result facilitates an increased amount of root exudation that provides supplementary nutrients to sustain the rhizosphere bacteria (Glick 2012).

In spite of iron being one of the most abundant elements on Earth, it is not readily assimilated both by bacteria and plants, hence making them iron deficient (Ma 2005). In the rhizosphere, where both microorganisms and plants compete for iron, bacteria synthesize certain compounds in order to sequester iron (Guerinot and Yi 1994). Siderophores are low molecular weight compounds known to chelate iron as well as other metals in soil, produced by a variety of microorganisms (Loper and Buyer 1991). They play an important role in heavy metal phytoextraction as they are able to solubilize iron by complexation (Gray and Smith 2005). Siderophores from PGPB can prevent some phytopathogens from acquiring a sufficient amount of iron, thereby limiting their ability to proliferate (Schippers et al. 1987).

The fallow phases help in renewal and enrichment of different soil properties along with an increase in soil microbial biomass and hence its diversity (Pandey et al. 2010). The survival of the plant growth promoters under shifting cultivation environments and their regeneration in *jhum* fallows is important for the health of cultivated crops.

3.4.1 Plant Growth-Promoting Fungi (PGPF) in *Jhum* Agriculture

The fungal isolates obtained from a *jhum* site in northeastern India were able to tolerate elevated temperatures and grow over a wide range of pH and salinity conditions. Many isolates also tested positive for various enzyme (pectinase, xylanase, cellulase) activity and were also able to show antimicrobial activity against pathogenic strains of *Alternaria alternata* and *Fusarium oxysporum*, suggesting them as potent candidates for use as bioinoculants in agriculture (Jain et al. 2016). Microorganisms characterized as plant growth promoters are known to aid plant growth by exhibiting traits such as solubilization of phosphates, production of enzymes, synthesis of auxin compounds, and siderophore production (Sharma et al. 2014). Phosphates are present in bound forms in soil and hence not available to plants but some microbes are able to solve this deficiency by solubilizing phosphate in soil (Khiari and Parent 2005). A number of bacterial and fungal isolates also produce auxin compounds like indole-3-acetic acid which help in cell elongation and cell proliferation in shoot and root of plants, thus enhancing growth (Tsavkelova et al. 2006). Certain species of fungi under the genera *Penicillium* and *Trichoderma* are known to promote the vegetative growth of plants by exhibiting such traits reducing the application of chemical fertilizers in agricultural soils (Sarma et al. 2015; Gupta et al. 2016). Soil fungi native to jhum were investigated for different PGP traits and tested for their efficiency in growth promotion of rice plant. The isolates were screened, and three were selected, namely, *Metarhizium pinghaense*, *Penicillium virgatum*, and *Penicillium stratisporum*, which exhibited promising effects improving shoot and root length of the tested rice plant (Banerjee et al. 2017). Such beneficial fungi need to be harvested and characterized further for them to be used in improving the sustainability and fertility of soil. Deeper research using advance technologies are required in order to have a better understanding of the integral aspects of *jhum* cultivation.

3.5 Application of the Plant Growth Promoters

The use of efficient and potential inoculants can be considered an important strategy for ecological management and for reducing environmental hazards by decreasing the use of chemical fertilizers (Alves et al. 2003; Balasubramanian et al. 2004; Hungria et al. 2010, 2013). Bacteria have different parameters or properties that influence plant growth and hence play a dynamic role in sustainability of agriculture through assimilated nutrient supply (Rengel and Marschner 2005). The efficacy and use of PGPRs and PGPFs as inoculants depend on the ability of these isolates to colonize the plant roots, interacting in the rhizosphere rich in root exudates and soil health (Danhorn and Fuqua 2007; Meneses et al. 2011; Alquéres et al. 2013; Beauregard et al. 2013). The colonizing efficiency of plant growth-promoting isolates of the plant roots is a closely related affair of the microbial competition and their survivability in the soil (Esitken et al. 2010; Lim et al. 2010). This also depends on the functional expression of several quorum sensing-related genes that help in cell

to cell communications. The plant root secretions differ under different environmental conditions which in turn affect the microbe-root surface interaction resulting in the adeptness of the inoculants (Bais et al. 2004; Cai et al. 2012; Carvalhais et al. 2013). Soil type, nutrient pool and toxic metal concentrations, soil moisture, microbial diversity, and soil disturbances caused by management practices affect soil health which is another important factor that affects the inoculation efficiency (de Souza et al. 2015). The microbial population is uneven throughout the soil, and reports suggest that shifting cultivated lands show a considerable decrease in the microbial community, as well as a loss of certain species of both bacteria and fungi, as compared to native forest lands (Miah et al. 2010). In a study carried out to see whether the native microbes inherent to the shifting cultivated fields of Northeast India could promote plant growth in the local upland variety of rice, both bacterial and fungal isolates were proved as promising growth enhancers. This was depicted by an in vitro plate assay and pot experiment with the rice seeds treated with the microbial inoculum that showed positive plant growth-promoting properties. For consideration as potent bioinoculants, the microbial isolates exhibiting PGP abilities were tested for early growth promotion of rice seed on plates. The microbial isolates used in a preliminary plate assay of seed germination revealed that the isolates brought about greater seed germination than that in the control. A total of six potent isolates, three bacterial and three fungal, were selected from a total of 87 screened isolates. The closest homologs of bacterial isolates like SB9 were *Curtobacterium oceanosedimentum*, *Bacillus methylotrophicus*, and *Bacillus cereus*, and the closest homologs of fungal isolates were found to be *Penicillium virgatum*, *Metarhizium pinghaense*, and *Penicillium stratisporum* (Banerjee et al. 2017).

The bacterial isolate *B. cereus*, which displayed the highest indole production, was able to induce the formation of the longest roots in accordance with most studies as the majority of the bacteria have been found to produce phytohormone IAA, although bacteria producing other cytokinins and gibberellins have also been reported (Ortíz-Castro et al. 2008; Morrone et al. 2009; Merzaeva and Shirokikh 2010). The fungal isolates, *Penicillium stratisporum*, showed the highest growth increase with comparable phosphate solubilization and indole production, while *Penicillium virgatum* exhibited the highest phosphate-solubilizing capacity with the greatest shoot length. 1–50% of the total bacterial population in soil constitutes of bacterial phosphate solubilizers much higher than that of fungi (for which the percentage is 0.1–0.5%) accounting for a higher number of cultured bacterial phosphate solubilizers (Zaidi et al. 2009). Most soils lack available phosphorus as one of its constituents and its availability is compromised when it forms insoluble salts with other inorganic compounds in soil (Vassilev and Vassileva 2003). Microbial isolates displaying IAA production increased root surface area and length (Vessey 2003), which in turn increases access to soil nutrients.

The potent isolates when tested in actual field trials gave satisfactory results in accordance with the pot experiments. There was a considerable difference in rice growth in the treated to that of control by the combined action of *Bacillus methylotrophicus* and *Bacillus subtilis* proved to be significant in case of the yield (Figs. 3.5 and 3.6).

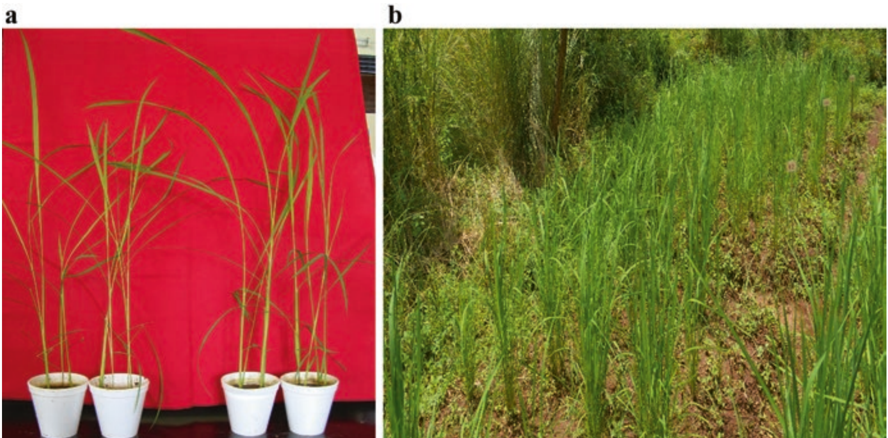

Fig. 3.5 Rice growth observed on pot (**a**) and in field conditions (**b**) using native bioinoculants from *jhum* system

Fig. 3.6 Rice (upland variety) cultivation in a *jhum* field

The practice of the addition of microbial inoculums as fertilizers in *jhum* fields is not widespread; hence, the use of inherent PGPs for enhancing crop output and supporting the eco-restoration and sustainability of the degraded conditions in *jhum* is a boon to the system. The natural phenomena of plant-microbe interactions are a necessary field to be researched in terms of its applications in order to increase the agricultural productivity and methods to be developed to advance their performance in the field, especially under extreme environmental conditions like those in a shifting cultivation system.

3.6 Conclusions

One of the promising techniques that has come up in recent times is the application of beneficial microbes both bacteria and fungi to agricultural systems for increasing crop productivity with improved biodiversity and soil health. Such interventions hold potential to serve as a solution to the rising problem of low productivity of crops (Bagyaraj and Ashwin 2017). This crisis currently being tackled by increased application of chemical fertilizers is, however, taking a toll on the biodiversity of agricultural ecosystems (Lupwayi et al. 2010). In order to assess the role they play in soil improvement, it is necessary to investigate the structure analysis and biodiversity indices of the soil-dwelling microbes (Frac et al. 2018).

In the northeastern part of India, the hilly topographies of the land with the prevailing climatic conditions, along with its diversified bio-resources, provide an appropriate milieu for the cultivators to practice *jhum* making it an intimate part of their lifestyles. To sustain the agricultural system for crop production, proper soil management strategy is necessary that can reduce soil degradation and improve soil quality and soil health. A deeper insight into the soil fungal biodiversity could be pivotal as it determines the thriving plant biodiversity and productivity in an agricultural ecosystem (van der Heijden et al. 1998; Wagg et al. 2014). Robust techniques are required for screening, harvesting, and harboring the populations of useful soil fungi in complex ecosystems for a sustainable plant production and restoration of a degrading environment. On a global scale, novel strategies need to be developed for the management and bioprospection of beneficial soil microbes for better cropping efficiency to overcome the challenges that pose a threat to food security today (Table 3.3).

Table 3.3 Reports on shifting cultivation from the northeastern part of India

Sl. no	Title	Authors	Year	Work done related to shifting cultivation
01	Soil nutrient status of hill agroecosystems and recovery pattern after slash-and-burn agriculture (*jhum*) in northeastern India	Ramakrishnan and Toky	1981	The study targeted the changes in fertility in agroecosystems where vegetation is removed by slash-and-burn procedures. The results showed a change in a number of physicochemical properties of soil pertaining to a net loss of carbon and other mineral components
02	Evaluation of bamboos in eco-restoration of "*jhum*" fallows in Arunachal Pradesh: ground vegetation, soil, and microbial biomass	Arunachalam and Arunachalam	2002	Analysis of the ground vegetation, soil, and microbial properties underlying the covering of three bamboo species, viz., *Bambusa nutans*, *Bambusa arundinacea*, and *Dendrocalamus hamiltonii*, showed mixed herbaceous vegetation with the variety *B. nutans* being recommended for rehabilitation of the *jhum* fallows due to highest microbial biomass nitrogen that was obtained from its surrounding soil

(continued)

Table 3.3 (continued)

Sl. no	Title	Authors	Year	Work done related to shifting cultivation
03	Ecosystem restoration of *jhum* fallows in Northeast India: microbial C and N along altitudinal and successional gradients	Arunachalam and Pandey	2003	A year old *jhum* fallow had greater herbaceous vegetation, while woody plants were observed in middle-aged fallows (7–16 years) with highest density in the forest. The study suggested that the altitudinal and successional dynamics of microbial C and N were linked to soil organic matter and total nitrogen contents in the soil during microbial community development after land abandonment from shifting cultivation
04	Changes in microbial biomass and activity in relation to shifting cultivation and horticultural practices in subtropical evergreen forest ecosystem of Northeast India	Ralte et al.	2005	The study conducted in the Nokrek biosphere reserve of Meghalaya in Northeast India analyzed the impact of human activities such as shifting agriculture and horticultural practices on temporal and spatial changes in microbial biomass. Microbial biomass C and N were found to be greater in the surface soil as compared to the subsurface soil layer. The steady increase in MBC and MBN and other enzyme (dehydrogenase and urease) activities from the young to the old fallows of *jhum*
05	Recovery of *Bacillus* and *Pseudomonas* spp. from the "fired plots" under shifting cultivation in Northeast India	Pandey et al.	2011	Soil from burnt plots of *jhum* lands in Arunachal Pradesh showed the bacterial communities were most affected along with the fungi and actinomycetes. The obtained bacteria reported belonged to species of *Bacillus* and *Pseudomonas*, namely, *B. clausii*, *B. licheniformis*, *B. megaterium*, *B. subtilis*, *B. thuringiensis*, *P. aeruginosa*, and *P. stutzeri*
06	Shifting cultivation: coexistence of forest and agriculture	Thakuria and Sharma	2014	Ecological traits of the plant population in the fallows of *jhum* stand as the impetus to the immediate microbial communities in soil allowing longer fallow length in rejuvenating the biological interactions over time. A microbial population and metagenomic work were a preface to a disturbed ecosystem in shorter fallows compared to that of longer fallows

(continued)

Table 3.3 (continued)

Sl. no	Title	Authors	Year	Work done related to shifting cultivation
07	Impact of shifting cultivation on litter accumulation and properties of *jhum* soils of Northeast India	Saplalrinliana et al.	2016	A recent study on the impact of aboveground biological inputs on the soil processes in various fallows of *jhum* depicted that longer fallow phase maintained not only higher quantity of accumulated forest floor litters but also sustained higher soil nutrient availability and restored state of C, N, and P cycling as evident from higher activity of soil enzymes
08	Native microorganisms as potent bioinoculants for plant growth promotion in shifting agriculture (*Jhum*) systems	Banerjee et al.	2017	Microorganisms native to shifting cultivation were isolated and screened for plant growth promotion (PGP) properties

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4 Diversity in Type III Secreting Systems (T3SSs) in Legume-Rhizobium Symbiosis

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Abstract

During nodule development, at least three sets of signals are exchanged between a legume host and its rhizobial partner. Apart from flavonoid and *nod* boxes, the third set of products are proteins exported by the type three secretion system (T3SS), which are necessary for continued infection thread development. The presence of active T3SS and its control of nodulation have been observed in *Bradyrhizobium japonicum* USDA110, *Sinorhizobium fredii* USDA257, *Rhizobium sp*. NGR234. However, the absence of active T3SS in the genomes of *Rhizobium leguminosarum* and *Sinorhizobium meliloti* argues against these effectors being modulators of nodulation. It is likely that alternative modulators exist, such as surface polysaccharides, which have similar or complementary roles to those proposed for effector proteins. Whether a secretion system facilitates symbiosis depends on both the legume and the bacterium, similar to what is seen in plant-pathogen interaction.

Keywords

T3SS · Legume-rhizobium symbiosis · Nodulation · Effectors

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4.1 Introduction

Diverse microorganisms are associated with plants in terms of epiphytes and endophytes and resulted in different kinds of ecological interactions ranging from mutualistic, pathogenic, and commensal in nature. Symbiotic microbial interactions were evolved to overcome the nutrient scarcity of plants in the new environment when habitat transition of plants into terrestrial from aquatic habitat occurred. Arbuscular mycorrhizal (AM) symbiosis and root nodule (RN) symbiosis are the two important mutualistic interactions studied extensively. AM symbiosis is evolved during early Devonian period, that is, 400 million years ago and nearly 80% of all terrestrial plant families are interacting symbiotically with AM fungi of glomeromycota (Kistner and Parniske 2002; Bonfante and Genre 2010). Hence, AM symbiosis is considered as the mother of plant root endosymbioses that helps to absorb minerals including phosphate via extraradical hyphae and supply them inside root cells via highly branched structures known as arbuscules (Harrison 1998; Parniske 2008). Certain soil bacteria collectively known as rhizobia symbiotically interact with legume plants and reduce atmospheric N_2 into chemical forms assimilated by host plant metabolism. Exchange of signal elements through nod-based signal transduction pathway which shares many elements with AM symbiosis is prerequisite for the initiation of such symbiotic interactions (Parniske 2008). Similarly, pathogenic microbial interactions imposed selection pressure and resulted in the development of sophisticated mechanisms in plants to respond to microbial infections. Each plant cell possesses pattern recognition receptors (PRRs) in plasma membrane to recognize the evolutionary conserved microbial molecules termed microbe or pathogen-associated molecular patterns (MAMPs/PAMPs) and induce the first tier of the plant immune system termed PAMP-triggered immunity (PTI). Jones and Dangl (2006) proposed "zigzag" model of pathogen– plant interactions, in which successful pathogens acquired the ability to overcome PTI by delivering proteins called effectors from bacterial cytosol into the host cytoplasm, resulting in effector-triggered susceptibility (ETS). Similarly, genes related to plant defense are initially upregulated in *Lotus japonicus* and *Medicago truncatula* after inoculation with rhizobia, then declined as nodules develop and hence the rhizobia is termed as an intelligent pathogen (D'Antuono et al. 2008; Jones et al. 2008).

Plant-associated bacteria export effector proteins across the membranes and cell wall using a variety of secretion systems to establish cross-kingdom communications and further effective colonization. These independently evolved secretion systems are containing different sets of core proteins. Flagella derived type III secretion system (T3SS) or conjugation apparatuses (type IV secretion system-T4SS) translocate effectors from the bacterial cytoplasm to the plant cytoplasm (Wooldridge 2009) while T1SS, T2SS, and T5SSs simply transport the effectors outside of the bacterial cell. Contact-dependent translocation of effectors through T3SS into host cells was first reported in *Yersinia pestis.* Since then, studies have established that bacteria use T3SS to communicate with protists, fungi, plants, and animals. Application of genomics to legume-rhizobium interactions revealed the presence of such protein secretion systems in rhizobia. Sequencing the plasmid pNGR234a of

Sinorhizobium fredii NGR234 and further analysis established the concrete evidence for the existence and role of T3SS in rhizobia (Viprey et al. 1998). Several studies on genome/plasmid sequencing revealed that T3SSs are distributed in several, but not all, rhizobial species (Kaneko et al. 2002; Göttfert et al. 2001; Krause et al. 2002; Krishnan et al. 2003; de Lyra et al. 2006). Propinquity is prerequisite for such secretion systems to inject effector proteins into their eukaryotic hosts. As rhizobia enter root hairs, intimacy with the plant cell is guaranteed and rhizobial T3SS genes are expressed in profoundly curled shepherd's crooks, infection thread, and developing nodules. Our knowledge of the molecular interplay between plant and microbial symbionts has developed over the past few decades. Commonalities on cellular signaling networks governing legume symbioses with rhizobia and mycorrhizae suggested that the molecular components of the legume-specific networks are shared with other plant-associated microbes. A deeper understanding of plant-associated microbial communities and their signaling pathways offers exciting opportunities to maintain crop health. In this chapter, we introduce the structure and diversity of rhizobial T3SS and their functional role in cross-kingdom signal transduction for host specificity and effective establishment of legume-rhizobia symbiosis.

4.2 Formation and Assembly of Type-3SS-Based Secretary Nanoapparatus in Rhizobia

Bacteria display multiple modes of host plant invasion including tissue damage; subvert the host immune response and protein secretion across phospholipid membranes. The protein secretion systems in bacteria play a stellar role in various plant – microbe interactions including pathogenic, commensalistic, or mutualistic association. These secretary proteins, which are also known as effector molecules, virulence factors or toxins, are produced in the cytosol of the pathogens and symbionts and passed across the bacterial cytoplasm into the host cytoplasm. Bacteria possess diverse protein secretion systems via Type I Secretion System-TISS, TIISS, TIIISS, TIVSS, TVSS, TVISS, and TVIISS. In TISS, TIIISS, TIVSS, and TVISS machinery, the secretary proteins are translocated across inner and outer membranes through a single step process whereas in TIISS and TVSS, proteins are exported by a two-step process in which molecules are first transported to periplasmic space *via* a universal sec or twin-arginine translocation (Tat) pathway (Fig. 4.1). Secretion *via* the sec pathway generally requires the presence of an N-terminal signal peptide on the secreted protein. In the sec pathway, the secreted proteins are translocated across the cytoplasmic membrane in an unfolded state. In the Tat pathway, a conserved secretion system to export fully folded proteins across the membrane of two consecutive arginine residues in the signal sequence are required to target the delivery. In addition to these, a specific TVIISS was identified in gram-positive mycobacteria that export proteins across the membrane and cell wall by one-step or two-step process (Tseng et al. 2009).

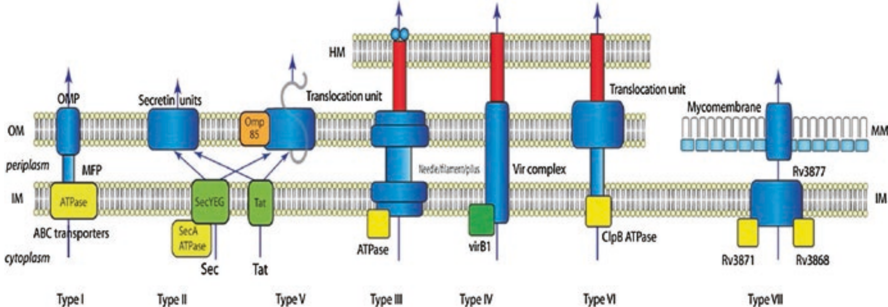

Fig. 4.1 Bacterial secretion systems (*HM* host membrane, *OM* outer membrane, *IM* inner membrane, *MM* mycomembrane, *OMP* outer membrane protein, *MFP* membrane fusion protein). ATPases and chaperones are shown in yellow. (Source: Tseng et al. 2009)

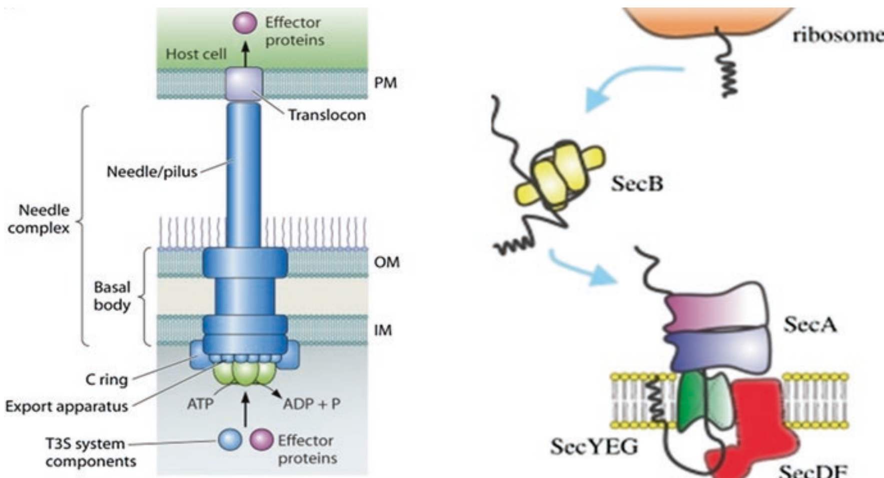

Fig. 4.2 Structural components of T3SS and protein secretion pathway of *E. coli*

4.2.1 Structural Components of Bacterial T3SS

Nonflagellar T3SS are evolved from the flagellum and eight out of nine core proteins of T3SS share a common ancestor with the flagellar apparatus (Burkinshaw and Strynadka 2014). The NF-T3SS, often referred to as injectisome, displays a needle and syringe like structure. It is a complex protein structure consisting of a multiring basal structure that spans the inner and outer bacterial membranes and ATPase complex in the cytosol (Fig. 4.2). All functional T3SSs proteins are grouped into nine families, viz., SctC, SctJ, SctN, SctQ, SctR, SctS, SctT, SctU, and SctV (Abby and Rocha 2012). The proteins conserved across T3SSs include outer membrane protein (SctC), export apparatus proteins (SctR, SctS, SctT, SctU and SctV), inner membrane ring proteins (SctD and SctJ), cytoplasmic ring protein (SctQ), and

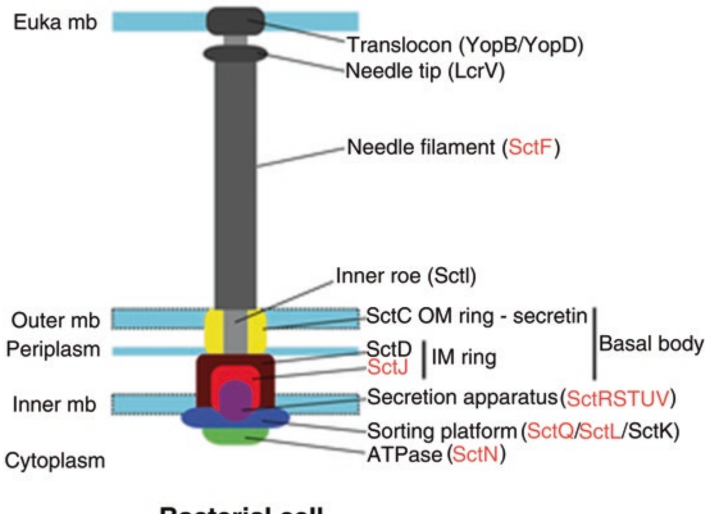

Fig. 4.3 Nonflagellar type III secretion system (NF-T3SS). (Source: Abby and Rocha 2012)

ATPase (SctN) (Wang et al. 2012). Proteins involved in T3SS function, but being less conserved, include the needle subunits (SctF and SctI), proteins encoding translocon (YopB and YopD), tip complex (LcrV), needle assembly (SctK, SctL, SctO and SctP), and effector export regulation (YopN) (Fig. 4.3; Table 4.1).

The secretion machinery (export apparatus) is attached to the basal unit. The basal ring at the inner bacterial membrane contains two secretion and cellular translocation proteins (SctJ and SctD) and the outer membrane is composed of SctC. The inner rod-like structure (needle/filament) extending to the eukaryotic host cell membrane is joined with a basal ring through the secretin, a channel forming proteins. A lipoprotein (YScW) facilitates the proper insertion of secretins in the bacterial outer membrane. At the distal end of the needle, LcrV controls the secretion and translocation. When outer needle sense host, bacteria secrete effectors and translocon (YopB and YopD) components which are surrounded by lipid vesicles. The translocon and tip proteins form a translocation channel (pore) in the host cell membrane through which effectors passes through. The needle has an inner hollow core that allows the unfolded proteins to traverse across the bacterial (inner periplasmic and outer extracellular spaces) and a host cell membrane in an ATP-dependent manner (Deane et al. 2006; Demers et al. 2014). In animal pathogens, SctF is the major subunit of needle complex, whereas in phytopathogens pilus-forming protein structure (forms channel) coded by HrpA. Both SctF and HrpA shares homology; however, pilus may be more adapted to a thick plant cell wall. Many of the TTSS proteins require chaperones that guide them to the needle complex of TTSS. Chaperons can protect the proteins from aggregation and degradation. A functionally interchangeable class IB chaperone of one bacterium can bind with effectors produced by other species. When the proteins leave the cytoplasm, chaperons detach from them and act as a transcription factor that induces the synthesis of more effector proteins.

Table 4.1 Essential components of T3SS

Ultra-conserved components					
Protein	**Component/Function**	**InterPro_Acc**[a]	**TIGRFAMs_Acc**[b]	**CDD_Acc**[c]	**EggNOG_Acc**[d]
SctC	Out membrane ring	IPR003522	TIGR02516	274174	ENOG4105K44
SctD	Inner membrane ring	IPR012843	TIGR02500	274166	ENOG4108SN3
SctJ	Inner membrane ring	IPR003282	TIGR02544	295084	ENOG4107Y59
SctN	ATPase	IPR005714	TIGR01026	273401	ENOG4108JIR
SctQ	Cytoplasmic ring	IPR013385	TIGR02551	274196	ENOG41069RK
SctR	Export apparatus	IPR005838	TIGR01102	130172	ENOG4108HHI
Scts	Export apparatus	IPR006306	TIGR01403	227131	ENOG41084IW
SctT	Export apparatus	IPR006304	TIGR01401	130468	ENOG4108TN6
SctU	Export apparatus	IPR029025	TIGR01404	130471	ENOG4108IUF
SctV	Export apparatus	IPR006302	TIGR01399	273600	ENOG4108HR8
Components less-conserved in composition or sequence					
Protein	**Component/Function**	**Representative InterPro_Acc**[a]		**UniProt_Acc for *Yersinia* representatives**[e]	
SctF/l	Needle subunit	IPR011841/IPR012670		Q7BRZ6/Q7BRZ3	
SctK/L/O/P	Needle assembly	IPR013388/IPR012842/1P R009929/I PR013354		Q7BRZ1/Q7BRZ0/Q9ZA80/Q9ZA79	
YopN	Effector export	IPR013401		Q93KT8	
YopB/D	Translocon	IPR013386		P37131/P37132	
LcrV	Tip complex	IPR005413		Q93KU4	

[a]InterPro_Acc, accession of protein family in the InterPro database (http://www.ebi.ac.uk/interpro/)
[b]TIGRFAMs_Acc, accession of protein family in the TIGRFAMs database (http://www.jcvi.org/cgi-bin/tigrfams/)
[c]CDD_Acc, accession of domain cluster in the CDD database (https://www.ncbi.nlm.nih.gov/cdd/)
[d]EggNOG_Acc, accession of protein otholog cluster in the EggNOG database (http://eggnogdb.embl.de)
[e]UniProt_Acc, the protein accession in the UniProt database (http://www.uniprot.org)

4.3 T3SS Gene Clusters of Phytopathogens

T3SS genes coding for its structural components, regulators, chaperones, and effector proteins are colocalized within the genome. They are organized in operons and the number of ORFs ranging from 24 (*Xanthomonas* pathovars) to 27 (*Pseudomonas* pathovars). T3SS genes are located either in the locus of enterocyte effacement (LEE) of approximately 22–50 kb chromosomal pathogenicity islands (PAI) of enteropathogenic *E. coli* (EPEC), enterohaemorrhagic *E. coli* (EHEC), *Citrobacter rodentium,* or in large plasmids of *Shigella*, *Yersinia, Pantoea agglomerans,* and *Ralstonia solanacearum*.

Five groups of T3SS systems in well-characterized bacterial pathogens of plant and animal were reported initially as follows (Hueck 1998; Foultier et al. 2002; He et al. 2004):

(i) Inv/Mxi/Spa group (*S. flexneri* and *S. typhimurium* SPI-1),
(ii) Ysc (Plasmids of *Yersinia* spp. and *P. aeruginosa)*,
(iii) Hrp1 (*E. amylovora* and *P. syringae)* and
(iv) Hrp2 (*R. solanacearum* and *X. campestris)*
(v) Esa/Ssa (*E. coli* (EPEC), *Salmonella* SPI-2, and *Y. pestis*-chromosomal T3SSs)

T3SS of plant pathogens were classified further based on gene composition, arrangement, and transcriptional regulations into *hrp*/*hrc1* (*Pseudomonas* and *Erwinia*) and *hrp*/*hrc2* (*Xanthomonas*, *Ralstonia*, *Acidovorax,* and *Burkholderia*). However, depending on the strain, they either lack some of the listed genes or carry additional ones (Tampakaki et al. 2010). Further research revealed widespread nature of T3SSs with considerable variation in the distribution, number and type of T3SSs among species, serovars, and strains (Wang et al. 2012; Barret et al. 2013). Hence, a unifying nomenclature was developed to describe the T3SS core components with the prefix sct (secretion and cellular translocation), followed by the suffixes used in the *Yersinia* Ysc system.

Most of the type III effector (T3E) genes are not physically linked to the T3SS gene clusters (Lindeberg et al. 2008), but usually scattered in the flanking regions of T3SS clusters. Tripartite gene structure of *hrp*/*hrc* clusters in most of the *Pseudomonas syringae* pathovars comprises exchangeable effector locus (EEL), *hrp/hrc* gene cluster, and conserved effector locus (CEL) (Alfano et al. 2000). Genes encoding T3S effectors and many sequences related to mobile genetic elements are present in EEL. T3E gene content and structure differ among species/pathovars (Tampakaki et al. 2010).

Similar to phytopathogenic bacteria, T3SSs genes in rhizobia are clustered in 30–47 kb symbiotic islands of either chromosome or symbiotic plasmids (pSym), along with genes involved in nodulation and nitrogen fixation (Annapurna and Krishnan 2003; Krishnan et al. 2003; de Lyra et al. 2006). T3SS gene clusters in rhizobia are designated *tts* (type three secretions), genes encoding T3SS core components are *rhc* (*Rhizobium* conserved), and secreted proteins are Nops (nodulation outer proteins). Phylogenetic analyses of *rhc* revealed a distinct T3SS gene family called Rhc (Rhizobiales family) (Marie et al. 2001; Troisfontaines and Cornelis 2005).

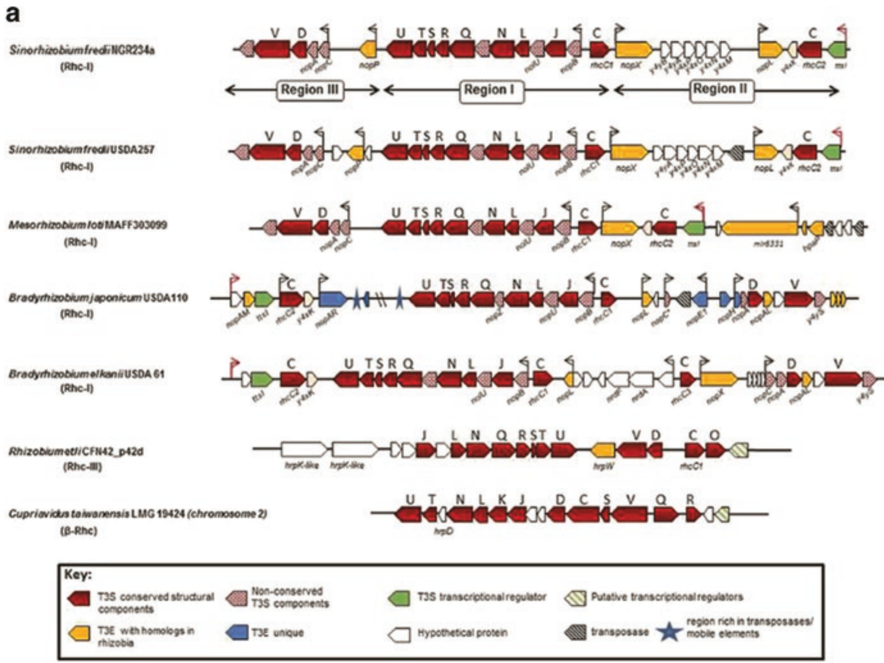

Fig. 4.4a Gene content and organization in T3SS of rhizobia

4.3.1 T3SS Gene Clusters of Rhizobia

Phylogenetic analysis of rhizobial T3SS core proteins resulted in further classification of Rhc into four subgroups (Gazi et al. 2012). Subgroup Rhc-I contains T3SS of *S. fredii*, *M. loti,* and *B. japonicum*, while the second T3SS located in plasmid pNGR234b of *S. fredii* NGR234 is classified under subgroup Rhc-II, and T3SSs of *R. etli* strains belong to Rhc-III (Fig. 4.4a, Table 4.2). A unique group designated β-Rhc is identified in β-rhizobium *Cupriavidus taiwanensis*. So far, only the *tts* clusters of Rhc-I group have been shown to influence the nodulation process.

4.3.1.1 T3SS Gene Cluster of Rhc-I

Genes encoding core components of T3SS in Rhc-I are organized into three distinct regions identified based on gene architecture, that is, a central region-I and regions-II and III, whose position is variable among the rhizobial species (Fig. 4.4a). The central region is highly conserved both in gene content and organization in all rhizobial species and constitute an operon with T3SS genes in the transcriptional order *nopB, rhcJ*, *nopU, rhcL*, *nopZ*, *rhcN, rhcQ*, *rhcR*, *rhcS*, *rhcT*, *rhcU* (Perret et al. 2003; Marie et al. 2004; Wassem et al. 2008). In addition, upstream of *nopB* is *rhcC1* in the opposite direction and without an obvious *tts* box in its upstream region.

Table 4.2 Distribution of Rhc-T3SS subgroups in rhizobia

	Rhc-I	Rhc-II	Rhc-III	β-Rhc
B. japonicum USDA110; *B. elkanii* USDA61; *B. elkanii* 587; *M. loti* MAFF303099	■			
Sfr NGR234; *Sfr* USDA257; *Sfr* HH103	■	■		
Smel KH12g; *Smel* KH48e; *Smel* M30; *Smel* M210; *Smel* M243; *Smel* M249; *Smel* M268; *Smel* T094; *S. saheli* USDA4893		■		
Smel GR4; *Smel* M195		■	■	
S. terrange USDA4894	■		■	
R. etli CFN42; *R. etli* CIAT652; *Rleg* bv. phaseoli 4292; *Rleg* bv. phaseoli FA23; *Rleg* bv. CCGE 510; *Rrhi* A4, *Rrhi*K84			■	
C. taiwanensis LMG 19424				■

Abbreviations*: S.,Sinorhizobium; M., Mesorhizobium; B. Bradyrhizobium; R. Rhizobium; Str, S. fredii; Smel, S. meliloti; Rleg, R. leguminosarum; Rrhi, R. rhizogenes; C., Cupriavidus; Colored boxes represent the presence of a T3SS gene cluster within a genome and the different colors correspond to the various Rhc subgroups*

T3SS gene content and organization in regions II and III of Rhc-I are variable and divergently oriented in *tts* clusters. These two variable regions are located down stream of *rhcC1 and rhcU*, respectively, in *Mesorhizobium loti* and *Sinorhizobium fredii*, but vice-versa in *Bradyrhizobium*. Genes such as *ttsI*, *rhcC2,* and *y4xK* coding for a putative lipoprotein are organized in region-II (Viprey et al. 1998; Marie et al. 2004). The *ttsI* of rhizobia formerly known as *y4xI* begins with a *nod* box and encodes T3SS-transcriptional regulator (Viprey et al. 1998; Krause et al. 2002; Marie et al. 2003, 2004; López-Baena et al. 2008; Sánchez et al. 2009). *nopC*, *nopA*, *rhcD*, *rhcV,* and *y4yS* are organized into an operon with a *tts* box in the upstream of *nopA* at region-III and no other discernible transcriptional motifs of the intergenic region between *nopA* and *rhcD* (Krause et al. 2002). The *tts* clusters in strains NGR234, USDA257, and HH103 belonging to *S. fredii* have identical genetic organization, highly conserved DNA sequences (98–99%) even in the intergenic regions, while the protein sequence similarities are poor and ranging between 94 and 100%. Apart from sequence variations, presence or absence of some genes in regions II and III resulted in minor diversifications of tts cluster belonging to Rhc-I (Fig. 4.4a).

4.3.1.2 Diversifications of T3SS Gene Cluster Within Rhc-I

Genes such as *y4yB*, *y4yA*, *y4xP*, *y4xO*, *y4xN*, and *y4xM* located between *nopX* and *nopL* of *Sinorhizobium* are absent in *Mesorhizobium and Bradyrhizobium* (Perret et al. 1999; Krishnan et al. 2003; Weidner et al. 2012). This gene cluster is

coregulated with *nopX* as predicted based on (i) no *tts* box in upstream region of *y4yB*, (ii) no transcriptional termination signals in the intergenic region between *nopX* and *y4yB*, and (iii) flavonoid-dependent expression of *y4xP* (Perret et al.,1999; Streit et al. 2004; Lorio et al. 2006). Production and secretion of Nops are not affected by the mutations in *y4yA*, *y4yB*, and *y4xP* (Jiang and Krishnan 2000; Lorio et al. 2006). Despite its location in T3SS locus, *y4xP encodes* cysteine synthase which is not a type III secreted protein. Interestingly, inactivation of *y4xP* in USDA191 resulted in enhanced nodulation in "McCall" soybean (Lorio et al. 2006). T3E genes such as *nopP* (*y4yP*) and *nopL* (*y4xL*) are considered as specific for *S. fredii* as their homologs are missing in *Mesorhizobium and* other phytopathogenic bacteria (Bartsev et al. 2004; Ausmees et al. 2004).

Region II *tts* cluster in *B. japonicum* possesses two additional ORFs upstream of the *ttsI* which are absent in other rhizobia (Fig. 4.4a). One is *nopAM,* a putative T3E gene and the other *bll1845* encodes an unknown protein (Kimbrel et al. 2013). The *nod* box does not reside upstream of *ttsI*, as in other rhizobial species, but upstream of *bll1845* (Krause et al. 2002). In addition, region-II or rest of the *Bradyrhizobium* genome is detected with neither *nopX* nor its homolog (Göttfert et al. 2001), but unique T3E genes such as *nopAR* (Yang et al. 2010; Kimbrel et al. 2013) and *bsr1831* are located (Zehner et al. 2008). This region is also rich in transposases and mobile elements, suggesting that may be the gene content has resulted from gene rearrangements.

Several unique ORFs were also reported in region III of *B. japonicum* USDA110. *nopL* encoding a smaller protein with 167 amino acids in comparison to that from *S. fredii* strains (338aa) is located downstream of *rhcC1* (Krishnan et al. 2003; Ausmees et al. 2004; Rodrigues et al. 2007). ORF-*bsl1808* coding for a hypothetical protein (67aa) is located downstream of the *nopL*-like gene, while *id211* coding for a polypeptide (91aa) are identified in region-III (Göttfert et al. 2001). Unique genes via *nopE1* (*blr1806*) coding for a characterized T3E protein (Wenzel et al. 2010; Schirrmeister et al. 2011), an ORF (*bll1805*) coding for a hypothetical protein, but without an obvious *tts* box in its upstream region and *nopH* (*blr1804*) coding for a putative T3E protein (Hempel et al. 2009; Kimbrel et al. 2013) were also located in region-III of *Bradyrhizobium*.

The plasmid-borne (pDOA9) T3SS gene cluster of *Bradyrhizobium* sp. DOA9 contains all the genes necessary for the formation of the secretory apparatus and the transcriptional activator (TtsI). The *tts* box is preceded by a *nod*-box motif. The genetic organization of secretion machinery is in the order of *nop*B, *rhc*J, *rhc*L, *rhc*N, *rhc*U, *rhc*Q, *rhc*R, *rhc*S, *rhc*T, *rhc*U (Fig. 4.4b). The mutation in T3SS showed the inability of *Bradyrhizobium* to establish symbiosis, however, its endophytic colonization in rice remains unaffected (Songwattana et al. 2017).

4.3.1.3 T3SS Gene Clusters of Rhc-II

Plasmid pNGR234b of *S. fredii* NGR234 possesses a second T3SS that belongs to Rhc-II group (Schmeisser et al. 2009). Similarly, two types of T3SS were detected in the plasmids of *S. fredii* HH103. Functional T3SS (T3SS-I) is located in

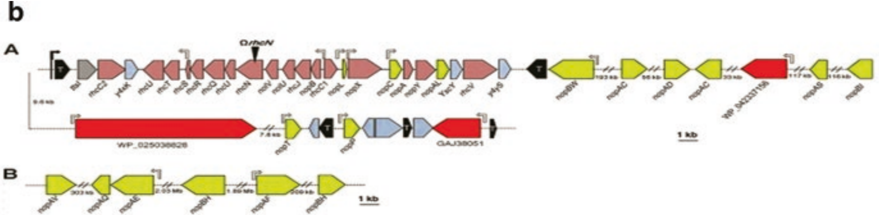

Fig. 4.4b Gene content and organization of T3SS genes in *Bradyrhizobium.* (**a**) T3SS in plasmid pDOA9 and (**b**) on the chromosome. The orientations and sizes of the ORFs are indicated by arrows. Location and orientation of *tts*-box motifs are shown by rectangular open arrows, while the *nod*-box motif is shown by a black rectangular arrow. The site of insertional mutation for Ω*rhcN* is indicated by a black arrowhead. (Source: Songwattana et al. 2017)

symbiotic plasmid pSfrHH103d (de Lyra et al. 2006; López-Baena et al. 2009) and a second one (T3SS-II) with genes encoding diverse surface polysaccharides is located in pSfrHH103e (Margaret et al. 2011). T3SS-II was also located in the chromosome of *S. fredii* USDA257 which is identical to gene content and genetic organization of strain NGR234. *tts* clusters of Rhc-II which is lacking typical regulatory elements of Rhc-I is detected so far only in *Sinorhizobium* (Table 4.2). Absence of *nod/tts* boxes in the promoter regions, weak transcriptional control of the major operon of T3SS-II cluster, independent of flavonoids and/or the presence of functional TtsI suggested the involvement of different regulatory elements for T3SS-II locus (Schmeisser et al. 2009). T3SS-II of *S. fredii* NGR234 contains 22 genes (NGR_b22800 to NGR_b23010) with 12 of them encode conserved T3SS components. All 22 genes (NGR_b22800-NGR_b23010) in T3SS-II or eight out of them were found to be significantly upregulated in bacteroids of indeterminate *Leucaena leucocephala* and determinate *Vigna unguiculata* nodules, respectively, compared with the free-living bacteria (Li et al. 2013). Differential level/fold upregulation of *nopP*, *nopX,* and *nopL* in bacteroids of *L. eucocephala* and *Vigna unguiculata* indicated that functionality of T3SS-II depends on either host or nodule type.

4.3.1.4 T3SS Gene Clusters of Rhc-III

Rhc-III group comprises the T3SSs of *R. etli, R. leguminosarum,* and *Agrobacterium rhizogenes* strains K84 and A4 (Setubal et al. 2009; Slater et al. 2009). However, complete genomes analysis of *A. tumefaciens* C58 (biovar1) and *A. vitis* S4 (biovar3) revealed the lack of T3SS. T3SS cluster of Rhc-III is detected on plasmid p42d of *R. etli* CFN42 and pB of *R. etli* CIAT652 (González et al. 2006; Gazi et al. 2012). Few core components of other T3SS clusters such as *rhcC2* were not detected on Rhc-III *tts* clusters. It also lacks *tts* boxes in the upstream regions of *rhc* genes and poor homology between its transcriptional regulator (RHE_PD00067) with TtsI suggests the unique way of T3SS regulation in *R. etli* strains. Interestingly, *hrpW* is highly conserved among diverse *R. etli* strains (Fauvart et al. 2009). Similar kind of T3SS gene content and their organization in two putative operons was detected in *R. leguminosarum* strains. In addition, genes coding for proteins similar to members of

StcC and SctO families and a putative transcriptional regulator COG1396 were also detected (Fig. 4.4b). Notably, gene content, organization, and phylogenetic position of T3SS in *A. rhizogenes* strains K84 and A4 are most similar, while some slight differences like the absence of gene coding for a member of SctO family are evident. In addition to lack of *hrpW,* a gene coding for protein (1060aa) low similarity to HrpK1 of *P. syringae* is detected.

4.3.1.5 β-Rhc Gene Clusters

T3SS genes of *Mimosa* nodulating β-rhizobium *C. taiwanensis* are organized into four operons in chromosome 2 without any effector genes. Genetic organization of this distinct β-Rhc gene cluster differs from all other rhizobial T3SS clusters and shares similarity with human opportunist *Burkholderia cenocepacia,* suggesting a common origin for bacterial secretion systems (Amadou et al. 2008). A recent study showed that, in contrast to α–rhizobia, glutamate rather than flavonoids induced T3SS expression in *C. taiwanensis* grown in minimal media (Saad et al. 2012).

4.3.2 Global Survey of Secretion System

A deep insight into the diversity, distribution of T3SS, and their unique nomenclature is the prerequisite to know their evolution and role on host-microbial interactions. Despite the high variability in the organization of T3SS core genes among the prokaryotes, Hu et al. (2017) revealed the presence of four microsynteny blocks (MSBs) using the comparative genomic approach with 36 well-characterized clusters curated in T3DB (T3 Data Base). Although the MSB covered most of the T3SS genes for core component and accessory proteins, a fifth conceptual MSB (MSB-5) described a variable cluster having genes encoding the translocon, tip complex, as well as adjacent chaperones (Fig. 4.5). Organization features of MSBs and their phylogenetic relationship resulted in the delineation of well-characterized T3SSs into 10 categories (I–X). MSB organization features captured by the supervising analysis were combined with phylogenetic analysis to recluster the 36 known T3SSs into 10 categories. T3SS clusters via. Bor, Des, and Vib-Chr2 are reported with unique gene content and/or synteny and categorized into their own distinct groups VIII–X, respectively (Hu et al. 2017). Hidden Markov Model (HMM) was built for each conserved gene within MSBs and nearly 20,000 prokaryotic genome sequences were subjected for mining T3SS genes. The study revealed the existence of 174 nonredundant T3SSs from 109 bacterial genera belonging to 5 phyla and their phylogenetic analysis expanded the T3SS categories into 13. Type-II T3SS of α-proteobacteria belongs to category-XI, T3SS of *Myxococcales* (δ-proteobacteria) belongs to category-XII, while unique T3SS which are not classified under any other categories, found in diverse *Proteobacteria,* are placed in category-XIII.

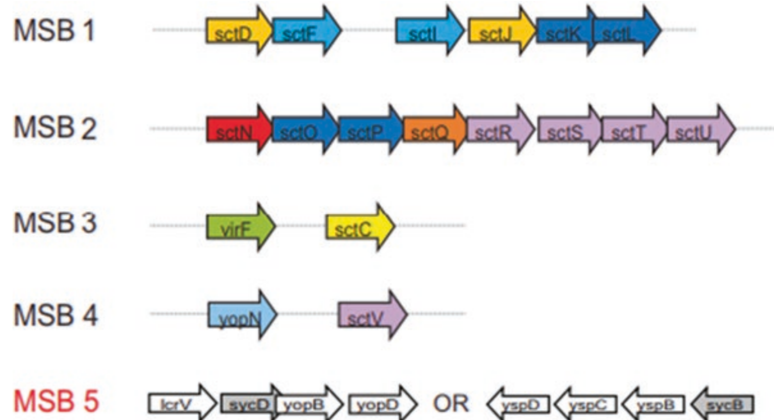

Fig. 4.5 Microsynteny blocks (MSBs) of T3SS

4.4 Regulation of Symbiotic Secretion Through T3SS

Signal molecules from the host are sensed and reciprocated by the bacteria using T3SS/T4SS secretion systems to determine and coordinate different stages of effective colonization. Any kind of environmental changes prevailing inside the host tissues such as temperature, pH, oxygen tension, extracellular Ca2+ concentration, and bile salts can trigger a complex regulatory pathway, resulting in binding of specific transcriptional regulators to conserved promoter elements of T3SS and T4SS genes (Fig. 4.6). Rhizobacteria are chemotactically attracted to host plants upon detection of signal compounds present in root exudates (Brencic and Winans 2005). For example, expression of the T3SS in *P. syringae*, a phytopathogen is induced by certain metabolites of susceptible plants, while are otherwise absent in resistant hosts. Rhizobial T3SS are induced by different groups of flavonoids and further resulted in the initiation of the nodulation process which is host specific in nature. First level determinants of host specificity are the type of flavonoid in the root exudates and NodD are protein induced by such flavonoids. NodD of rhizobia is the environmental sensor to interact with flavonoids in the rhizosphere, and act as master transcriptional activator for genes downstream of special promoters called *nod* boxes genes (nod, noe, nol, etc.) including those involved in the synthesis of Nod factors and protein secretion (Kobayashi et al. 2004; Marie et al. 2004; Wassem et al. 2008). Nod factors are lipochitooligosaccharides consisting of a conserved backbone that is varyingly decorated with accessory groups. Only particular types and mixtures of Nod factors allow a rhizobial strain to nodulate a certain legume host, thus giving rise to a first rhizobial determinant of host specificity (Spaink 2000). Localized accumulation of Nod factor molecules induced early responses including calcium spiking, modifications in root hair cytoskeleton, and further

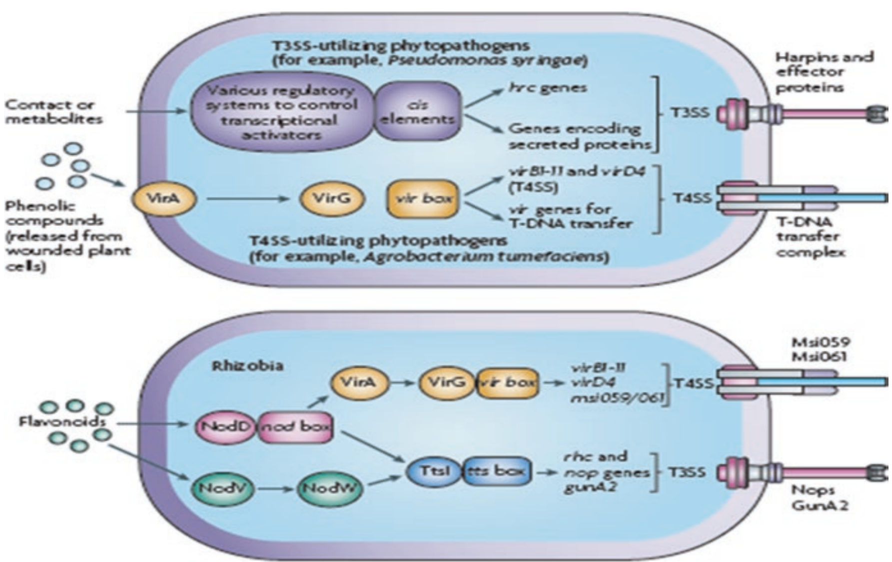

Fig. 4.6 Regulation of rhizobial protein secretion systems. (Source: Deakin and Broughton 2009)

Table 4.3 Glutamate activates T3SS genes of *C. taiwanensis*

	β-Galactosidase activity (SD) of construct in MM/4-S medium containing:			
Construct	No Inducer	Apigenin	Luteolin	Glutamate
pCZ388	25.8 (± 7.3)	18.5 (± 8.8)	20.2 (± 10.1)	28.9 (± 3.9)
pCBM01	89.6 (± 8.8)	2,689 (± 322)	2,287 (± 125)	128.0 (± 34.1)
pCZ-PsctV	35.4 (± 3.9)	17.3 (± 9.7)	32.4 (± 4.1)	591.4 (± 53.9)

curling of the root hairs. However, luteolin or apigenin, two inducers of LMG19424 nodulation genes such as *nodB* (Table 4.3) fail to induce expression of *C. taiwanensis* T3SS genes. In contrast, the addition of glutamate triggered the activity of P*sctV* and fail to induce *nodB*-lacZ fusion (pCBM01), confirming the flavonoid-independent regulation of T3SS genes in *C. taiwanensis.*

Second level determinants of host specificity are rhizobial surface cyclic glucans, polysaccharides like lipopolysaccharides, exopolysaccharides, and capsular polysaccharides. Role of rhizobial secreted extra cellular polysaccharides (EPS), lipopolysaccharides (LPS), capsular polysaccharides, and cyclic β-glucans in symbiosis was described in our earlier review (Senthilkumar et al. 2017). During infection thread development, correct composition of above polysaccharides interacts with the host plant and result in successful symbiosis. Cell division continues in nodule primordium and initiates RN development. After the release of rhizobial cells from infection thread into the cytoplasm of the host cell, they differentiate into bacteroids and fix nitrogen. In addition to Nod factors and surface polysaccharides, the third class of determinants called effector proteins are secreted by T3SS.

A regulatory motif termed as *tts* box generally located in the promoter regions for T3SS is considered as a binding site for the transcriptional activator TtsI. Flavonoid-dependent regulatory cascades control the expression of TtsI and both nodulation (*nod*, *nol*, *noe*) as well as T3SS (*sct*) genes (Viprey et al. 1998; Kobayashi et al. 2004; Krause et al. 2002). Transcriptional activator TtsI shares characteristic features with the DNA-binding response regulators of two component regulatory systems, but a mutated key amino acid, otherwise normally phosphorylated to activate response regulators, render the protein constitutively active (Marie et al. 2004). Its binding to specific *cis*-elements (*tts* boxes) found upstream of rhizobial T3SS cluster initiates transcription of T3SS genes (Wassem et al. 2008). Detection of conserved *cis*-elements in the upstream of T3SS and T4SS genes (Hubber et al. 2004; Krause et al. 2002) encouraged the comprehensive database mining for localization of other coregulated genes. For example, most of the genes in 11 functional tts boxes of pNGR234*a* are related to T3SS functions and others are controlling the expression of genes involved in plasmid stability and synthesis of lipopolysaccharides (Marie et al. 2004; Wassem et al. 2008).

There are only two *vir* boxes located in the symbiotic island of *M. loti* R7A. However, it was speculated that the others may exist elsewhere in the genome (Hubber et al. 2007). Huge variability was observed in the number of tts boxes in completely sequenced T3SS-possessing rhizobial strains. A total of seven *tts* boxes with genes predominantly associated with the T3SS locus and putative effectors are detected in *M. loti* MAFF303099, whereas at least 30 *tts* boxes are detected in the genome of *B. japonicum* USDA110 (Zehner et al. 2008; Süss et al. 2006). TtsI regulon of *B. japonicum* USDA110 and *Rhizobium* sp. NGR234 are not completely devoted to T3SS functions and several open reading frames downstream of *tts* boxes are speculated to encode novel rhizobia-specific effector proteins. Tts boxes are detected in promoter regions of genes unrelated to T3SS, such as those involved in the biosynthesis of rhamnose-rich polysaccharides (Marie et al. 2004) and led to unraveling the complex interplay between the T3SS and surface polysaccharides in the molecular dialogue of symbiotic process (Broughton et al. 2006).

4.5 Role of Rhizobial T3SS and Effector Proteins in Legume–Rhizobium Interactions

Propinquity is prerequisite for injecting effector proteins into the eukaryotic hosts through bacterial secretion systems. Such kind of intimacy with the plant cell is guaranteed as the rhizobia entrapped in "shepherd's crooks" through root hair infection delivered into plant cells via infection thread and results in nodule development. It was well established that T3SS genes are expressed at all of these stages. T3SS of *Bradyrhizobium japonicum* USDA110 was expressed in infection threads and developing nodules of *Glycine max (*Zehner et al. 2008), and transcripts of several T3SS genes of *Rhizobium* sp. NGR234 were detected in mature nodules of *Cajanus cajan* and *V. unguiculata (*Viprey et al. 1998; Perret et al. 1999).

Secretion system of *Rhizobium* sp. NGR234 is activated after the synthesis of Nod and continued for at least 24 h (Marie et al. 2004).

Sinorhizobium fredii USDA257 secretes several Nop proteins including NopX, NopL, and NopB into supernatants of flavonoid-induced cell cultures (Krishnan and Pueppke 1993; Annapurna and Krishnan 2003). Soybean cultivar-specific protein NopX is absent in Bradyrhizobium japonicum USDA110. NopX is homologous of HrpF of *X. campestris* pv *vesicatoria.* HrpF functions as a translocator of effector proteins into the host cells. It suggested that NopX may be involved in other functions in addition to mediating host specificity in soybean. Cultivar specific locus *nolXWBTUV* in *Rhizobium* NGR234 is detected in the T3SS gene cluster. Mutation at this locus affected symbiosis in a host-specific way as the mutant strains lost their ability to secrete proteins. Mutant studies indicated that T3SS of *Rhizobium* sp. NGR234 plays a positive role in *Tephrosia vogelii* where effective nodulation required secreted proteins, a negative role in *Pachyrhizus tuberosus* and *Phaseolus vulgaris* where nodules aborted during development and a neutral role where symbiosis was not affected in *Lotus* and *Vigna.* T3SS genes were subsequently detected in several rhizobial genera including *B. japonicum* USDA110, *Sinorhizobium fredii* strains HH103 and USDA257, *Mesorhizobium loti* MAFF303099, and *R. etli* CNPAF512 (Kaneko et al. 2000; Krause et al. 2002; Krishnan et al. 2003; Hubber et al. 2004; de Lyra et al. 2006). In every case, T3SS influence the symbiosis in a host-specific manner.

Complex macromolecular components of T3SS span the inner and outer membrane of the bacterial cell and host cellular membrane. T3SS secretes effector proteins into host cells for effective colonization, as well as helper proteins for assisting this translocation process. Effector proteins of rhizobial T3SS are termed as nodulation outer proteins (Nops) by Marie et al. (2001) to mimic the nomenclature of *Yersinia* outer proteins (Yops). Major and minor components of rhizobial T3SS are designated as helper proteins NopA and NopB, respectively, while NopX, another helper protein, constitutes the translocon structure in the cell membrane of legume host (Saad et al. 2005, 2008). Furthermore, *R. etli* possesses hrpW, homologous to the helper protein HrpW of *Pseudomonas syringae* (González et al. 2006) that promotes translocation of T3SS effector into plant leaves (Kvitko et al. 2007).

T3SS in Sinorhizobium fredii HH103 secretes three extracellular pili proteins (NopA, NopB, and NopX) and five putative proteins (NopC, NopD, NopL, NopM, and NopP). Effector proteins such as NopL, NopP, and NopC are exclusively present in rhizobia and influence the symbiotic process in a host-specific manner without affecting the secretion of other Nops (Marie et al. 2003; Ausmees et al. 2004; Skorpil et al. 2005; Dai et al. 2008; Deakin and Broughton 2009; Jimenez-Guerrero et al. 2015). Effector proteins NopL and NopP are generally phosphorylated by plant kinases and interfere with plant signaling pathways. NopL is phosphorylated by a Map kinase (Skorpil et al. 2005) and downregulates defense mechanisms of host legumes such as chitinases in *L. japonicus* (Bartsev et al. 2004). In *Rhizobium* NGR 234, NopL is known to delay nodule senescence (Zhang et al. 2011). The phosphorylation of NopL in *S. fredii* NGR234 is known to modulate

mitogen-activated protein kinase (MAPK) signaling (Zhang et al. 2011) and suppress premature senescence of nodules in *Phaseolus vulgaris*.

NopT is characterized as cysteine protease with myristoylation site and shared homology with YopT of *Yersinia* as well as Avr protein-AvrpphB of *Pseudomonas syringae* (Dai et al. 2008). Conserved catalytic triad of amino acids in C58 cysteine protease domain of this protein family determines their protease activity. Nearly 60 aminoterminal residues of AvrpphB are removed by autoproteolytic activity to reveal a myristoylation site that is further modified by host enzymes to target plasma membrane (Nimchuk et al. 2000). Site-directed mutagenesis revealed the importance of NopT's protease activity for host recognition and suggested the existence of RpS5-like surveillance system in other plants (Kambara et al. 2009).

Effector proteins of *S. fredii* T3SS such as NopD and NopM shared homology with XopD and Yop of *Xanthomonas* and *Yersinia*, respectively (Rodrigues et al. 2007). These effector proteins target the host cell nuclei and interfere with their protein regulation during colonization (Hotson et al. 2003). NopM belongs to the IpaH-SspH-YopM family of effectors lacking classical nuclear localization signals. XopD targets host cell nuclei with the hydrolysis of small ubiquitin-like modifier-conjugated proteins (Hotson et al. 2003), while YopM acts as a scaffold for recruiting and stimulating other proteins (McDonald et al. 2003). IpaH9.8 of *Shigella flexneri* is an E3 ubiquitin ligase (Rohde et al. 2007). NopM contains a conserved cysteine residue for ligase activity, while C48 cysteine protease domain of NopD involves in deubiquitination of eukaryotic proteins. However, XopD of *Xanthomonas* targets host proteins with small ubiquitin-like modifier termed as SUMO (Hotson et al. 2003).

T3SS-dependent secretion of NopC with unknown function and GunA2 with endoglucanase activity is reported (Süss et al. 2006). In *S. fredii* HH 103, *nopC* was located in the symbiotic island, and its expression was triggered by flavonoids. The inactivation on *nop*C inhibits nodulation in soybean (Jimenez-Guerrero et al. 2015). Effector protein yha00035 of *R. etli* shared homology with HopG1 of *Pseudomonas syringae* (González et al. 2006). Similar kind of homology-based gene mining significantly expands the pool of potential T3SS effectors involved in legume–rhizobium interactions. NopJ with C55 cysteine protease domain shared homology with YopJ family effectors of T3SS containing pathogens that target SUMO-conjugated proteins and interfere with MAP kinase signaling pathways (Orth et al. 2000). NopJ has recorded only the Avr-like negative effects on legume nodulation. Another homolog effector y4lO of *Rhizobium* sp. NGR234 is characterized as cysteine protease and reported to inhibit mitogen-activated protein kinase signaling pathways (Freiberg et al. 1997).

The absence of *nop*P improves the symbiotic potential of *S. fredii* HH103 in Williams soybean. NGR234 strain secretes eight T3SS proteins, and NodD1 and y4xI along with flavonoids regulate the transcription. Mutation in rhcN and y4xI confirmed that rhcN locus is required for flavonoid-induced NodD1 and y4xl-dependent secretion (Krishnan et al. 1995). In the NGR234, T3SS Nod box is located upstream of the two-component transcriptional regulator (y4xI).

NopP codes for T3SS effector in USDA257 (Kim and Krishnan 2014), while NopE1 and NopE2 code for effector molecules of USDA110 (Schechter et al. 2010). The NopX of *Sinorhizobium fredii* USDA257 and *Mesorhizobium loti* code for translocators shares homology with HrpF of pathogens. These translocators help in the transportation of the T3SS effectors into the host cell.

Detailed analysis of the effects of secreted proteins in *Rhizobium* sp. NGR234 has revealed that detrimental effects are often due to the action or recognition of a single negative effector like NopJ of *P. vulgaris* cultivars and NopT on *C. juncea* which are homolog to Avr proteins of phytopathogens. Plant beneficial effects are more complex as more than one effector protein is responsible. For example, NopL and NopP enhance nodule formation by *Rhizobium* sp. NGR234 in *Flemingia congesta*. Double mutant for *nopLnopP* has a similar phenotype to a T3SS knockout of strain NGR234 (Skorpil et al. 2005). The same double mutant is actually a poorer symbiont than the T3SS knockout of NGR 234 in *T. vogelii* and revealed the secretion of negatively acting effector into *T. vogelii* whose effects can be recognized only when the positive effects of Nopl and Nopp are abolished. Similarly, *Rhizobium* sp. NGR234 secretes a mixture of positively (NopM and Nopp) and negatively (NopJ) perceived effectors in *Lablab purpureus* and confirmed that overall beneficial effect of the T3SS on nodulation is a result of an equilibrium of the actions of various Nops (Kambara et al. 2009) (Fig. 4.7 and Table 4.4).

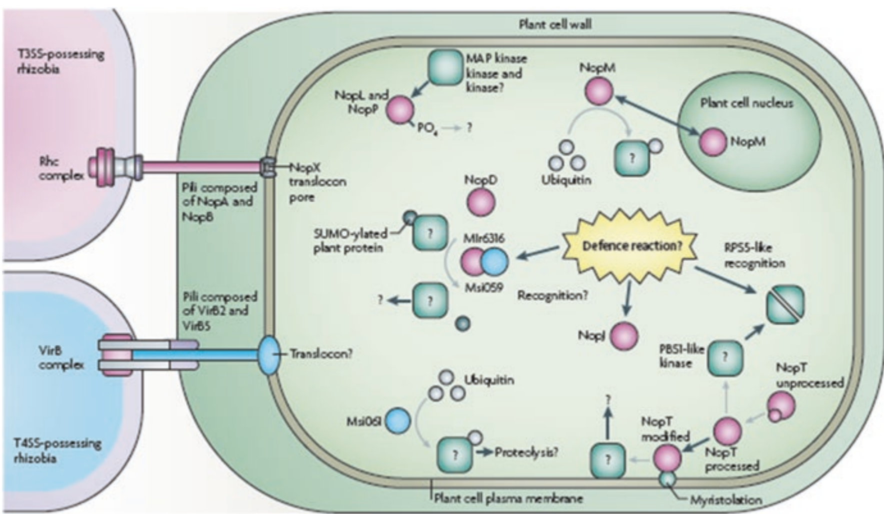

Fig 4.7 Functions of rhizobial effectors. (Source: Deakin and Broughton 2009)

Table 4.4 Symbiotic protein secretion systems

Species	Secretion system	Secreted proteins	Potentially secreted proteins	No significant effect on symbiosis	Positive effect on symbiosis	Negative effect on symbiosis
Bradyrhizobium japonicum USDA110	T3SS; symbiotic island	NopP, CunA2 (detected by MALDI-TOF-MS), Blr 1806, and Blr 1649	NopA-C, NopL, NopM, NopT: 30 tts boxes are predicted throughout the genome	*Vigna unguiculata*	*Glycine max Macroptilium atropurpureum*	None known
Mesorhizobium loti MAFF303099	T3SS; symbiotic island	Probably Mlr6316	NopA-C and NopX	*Lotus corniculatus*	None known	*Leucaena leucocephala*
Rhizobium sp. NGR 234	T3SS; symbiotic island	NopA-C, NopJ, NopL, NopM, NopP, NopT, and NopX	Several tts boxes upstream of potentially secreted proteins on symbiotic plasmids	*L. leucocephala L. japonicus V. unguiculata*	*Lablab purpureus, Flemingia congesta, V. unguiculata*	*Crotalaria juncea, Pachyrhizus tuberosus, Phaseolus vulgaris*
Rhzobium etli CNPAF512	T3SS	Although protein secretion was not detected, mutation of the system increased aberrant nodule-like structures on certain *P. vulgaris*	NopP and HrpW	None known	None known	None known
Sinorhizobium fredii HH103	T3SS	NopD, NopL, NopM, NopP, and NopX	NopB	*Cajanus cajan Crotalaria juncea V. unguiculata*	*G. max* cultivar Peking, Heinong33, kochi, Tribune and williams	*Erythrina variegata*
S. fredii USDA257	T3SS	NopX, Nop38, NopP, NopA, NopB	NopL	*V. unguiculata*	*G. max* cultivar Peking and *M. atropurpureum*	*G. max* cultivar McCall and *Erythrina* spp.

Source: Deakin and Broughton (2009)

4.6 Conclusion and Future Research

Bacterial protein secretion systems play a stellar role on the effective colonization of host tissues. Secretion systems in plant-associated bacteria are evolved independently with a different set of core proteins to export effector proteins for cross-kingdom communication with plants (Piromyou et al. 2019). Among these protein secretion systems, T3SS has been recognized particularly for its involvement in host-microbe interactions. Tip complex/translocon of T3SS pierces cell membrane of host plants and creates a conduit through which virulence/effector proteins (T3SEs) can be delivered directly into host cytoplasm which in turn cause a sequence of changes including subversion of host defenses.

Symbiotic nitrogen fixation is an important phenomenon of legume crops for fixing atmospheric nitrogen symbionts collectively termed as rhizobia. Sequencing of genomes and plasmids of rhizobia revealed that T3SSs are distributed in several, but not all, rhizobial species. The Rhc T3SS family is subdivided into four subgroups via Rhc-I (*S. fredii*, *M. loti,* and *B. japonicum*), Rhc-II (second T3SS of *S. fredii* NGR234 located on plasmid pNGR234b), and Rhc-III (*R. etli*). A distinct fourth group designated β-Rhc is identified in β-rhizobium *Cupriavidus taiwanensis*. Hidden Markov model (HMM)-based detection and categorization has reported 174 nonredundant T3SSs from 109 genera and 5 phyla, which cluster into 13 T3SS families. Recent studies revealed that T3SSs were even more widespread with considerable variation in the distribution, number, and type of T3SSs among bacterial species, serovars, and strains. Our understanding of the diversity and distribution of T3SS expanding with the increasing availability of new bacterial genome sequences and hence nomenclature and classification of T3SS needs to be frequently revisited.

To date, T3SS research has been focused on pathogenic bacteria, and hence efforts are needed in uncovering the specific structural and secretion regulatory components in T3SSs of symbiotic bacteria. Novel noncanonical T3SSs with yet unknown roles in both pathogenic and symbiotic bacteria revealed fascinating strategies exploited by the bacteria to interact with their host. Future research efforts should be directed to defining the signaling circuits that control the expression of the atypical T3SSs. Deciphering the regulatory networks will be the key to the future development of novel functional assays for screening and characterizing novel effectors.

T3SE effector protein encoded by *innB* is reported to determine the incompatibility of *Bradyrhizobium elkanii* USDA61 with mungbean and soybean (Nguyen et al. 2018). Identification of a large number of effector candidates and new insights into the possible transcriptional regulation of T3SS in multiple bacterial species may lead to the development of new strategies for increasing nodule formation. Moreover, this knowledge will contribute to the development of various biotechnological applications evolving efficient strategies to control plant diseases, the exploitation of T3SS as a scaffold for protein delivery into eukaryotic cells, the improvement of the symbiotic properties of rhizobial species, or even the expansion of the symbiotic potential toward nonleguminous plants of agriculture importance.

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Microbe-Based Novel Biostimulants for Sustainable Crop Production

5

Rahul Singh Rajput, Ratul Moni Ram, Anukool Vaishnav, and Harikesh Bahadur Singh

Abstract

The emerging status and scope of microbial products for better plant growth and prevention of diseases have attracted attention of researchers, industrialists to promote this field and farmers to utilize them as microbial stimulants. The hazardous impact of chemical fungicides in our ecosystem can also be mitigated through these strategies. Owing to the multifarious applications of biostimulants, agriculturally important microorganisms (AIMs) have been incorporated in agricultural system as biofertilizers and biopesticides. AIMs employed multiple mechanisms including nutrient solubilization, production of siderophores, phytohormone, antimicrobial compounds and volatiles, ACC deaminase and exopolysaccharide to work as biostimulant for alleviation of abiotic and biotic stresses in plants. In the present chapter, a comprehensive study on microbial biostimulants has been emphasized to confer their growth promoting and stress alleviation activities in plants. This would surely facilitate in a profound perception about mechanism of the plant-microbe interaction. Once a better knowledge developed about the governing action mechanisms of the microbe-based biostimulants is made, it will be easy to target next generation of biostimulants which may have multitargeted approach.

Keywords

AIMs · Biostimulants · Biofertilizer · Biopesticide · Plant growth promotion

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5.1 Introduction

The world's food supply for increasing population is a major challenge in the current scenario. To meet this demand, food production must increase approximately double till 2050 (Bruinsma 2017). Green revolution incited the use of fertilizers, pesticides and genetic modification to ensure the food supply for increasing population. Most of the fertilizers and pesticides are petroleum-based synthetic compounds. The excessive use of these compounds degrades soil health globally. Hence, further efforts must be emphasized on sustainable and eco-friendly manner. In this context, biostimulants are entirely natural and sustainable approaches that enhance plant growth by regulating plant physiological processes. Biostimulants are biological additives including humic acid, fulvic acid, seaweed extracts, protein hydrolysates, chitosan and beneficial microorganisms, which not only promote the plant growth and nutritional status but also induce tolerance against abiotic and biotic stresses in plants (Yakhin et al. 2017).

Among different biostimulants, agriculturally important microorganisms (AIMs) have received worldwide attention and acceptance for sustainable agricultural benefits. These microbes are increasingly being incorporated into crop production systems in order to optimize productivity without generating harmful side effects in the short or long term. Application of microbial biostimulants induce natural processes in soil like: sanitation, microbial biomass, soil structure and texture, nutrient content that ultimately enhance plant capacity for nutrient and water absorption and increase photosynthetic activity and tolerance level to environmental stresses (Fig. 5.1). In addition, AIMs are observed as efficient microbial competitors in the rhizosphere and rhizoplane region. They are also used for controlling several plant pathogens and rhizoremediation. These microbes are applied via seed treatment, soil application and foliar spray.

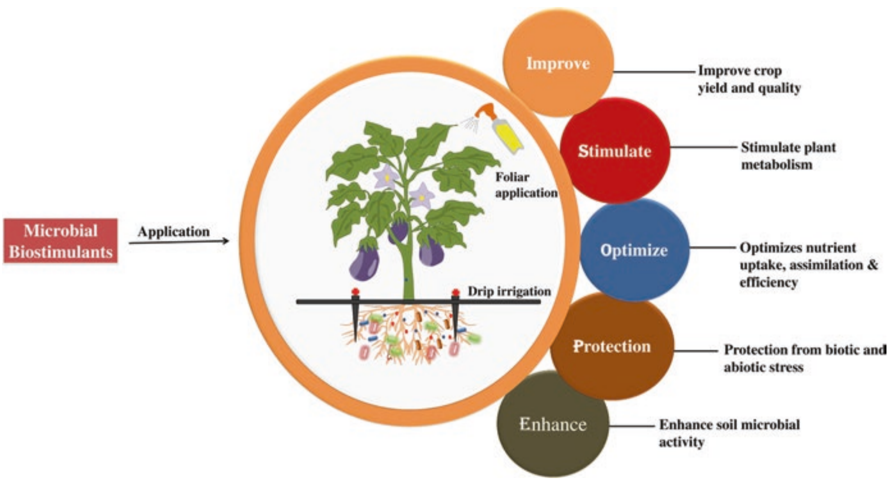

Fig. 5.1 Effect of biostimulants on different aspects involved in plant growth promotion

Our understanding of plant-microbe interactions has improved considerably since the last decade. The use of high-throughput technologies allowed us to gain more details about the microbial community in plant niches and their communication with their host and enabled us to learn how the microbes affect plant growth throughout the year. In reference, the present study describes plant-microbial biostimulants, especially their category and determinants, which are associated with plant growth promotion and amelioration of biotic and abiotic stresses.

5.2 Microbes as Biostimulants

In current agricultural practices, more efforts have been channelized towards the organic farming, in which production of 'enriched nutrient high-quality food' in sustainable ways has been mainly focused. Microbiological inputs in the form of biofertilizers and biopesticides have attracted lots of attention in organic farming. Among organic inputs, agriculturally important microorganisms (AIMs) occupy central position in biostimulants, which affect crop productivity, soil health and management of biotic and abiotic stresses under changing climatic conditions (Keswani et al. 2013; Mishra et al. 2015; Bisen et al. 2016). The application of AIMs as biostimulants minimizes the application of chemical fertilizers and pesticides during crop cultivation. These microbes increase the available form of nutrient contents in soil that enhance nutrient uptake efficiency in plants and show biocontrol activity that helps to protect plants against phytopathogens. In this section, we will highlight different aspects of microbes as biostimulants in agricultural practices.

5.2.1 Microbes Promoting Plant Growth

There are several bacteria, fungi, mycorrhizae, cyanobacteria and actinobacteria which enhance the plant growth and developmental activities through various mechanisms. These mechanisms include nitrogen fixation, solubilization of inorganic compounds, mineralization of organic compounds, plant hormones production, siderophore, ACC deaminase, antimicrobial compounds, HCN and hydrolytic enzyme production. In basis of their mode of action, PGPM can be subgrouped as biofertilizers, phytostimulators, biopesticides and bioprotector. PGPMs belong to several genera, viz. *Arthrobacter*, *Azotobacter*, *Azospirillum*, *Bacillus* spp., *Enterobacter*, *Pseudomonas* spp., *Rhizobium*, *Serratia*, etc. Among fungi, *Trichoderma*, *Aspergillus*, *Penicillium*, *Metarhizium*, *Beauveria* and arbuscular mycorrhizal fungi (AMF) are important (Choudhary et al. 2016).

5.2.1.1 Biofertilizers

Bacterial or fungal formulations are applied with a carrier material in agricultural field known as biofertilizers. Biofertilizers are easy to use and an economically and environment-friendly approach. Application of biofertilizers is done via seed or soil

Table 5.1 Microorganisms involved in plant growth promotion

S.N.	Group	Example
N_2 fixers		
1.	Symbiotic	*Methylobacterium*, *Rhizobium*, *Anabaena azollae*, *Frankia*
2.	Associative symbiotic	*Azospirillum* sp.
3.	Free living	*Azotobacter*, *Beijerinckia*, *Klebsiella*, *Burkholderia*, *Enterobacter*, *Clostridium*, *Anabaena*, *Nostoc*, *Herbaspirillum*
P solubilizing		
1.	Bacteria	*Bacillus*, *Pseudomonas*, *Rhizobium*, *Agrobacterium*, *Micrococcus*, *Burkholderia*, *Acetobacter*, *Flavobacterium*
2.	Fungi	*Trichoderma harzianum* T-22, *Aspergillus awamori*, *Penicillium* sp.
P mobilizing		
1.	Arbuscular mycorrhiza	*Gigaspora* sp., *Glomus* sp., *Sclerocystis* sp., *Scutellospora* sp., *Acaulospora* sp., *Rhizophagus* sp.
2.	Ectomycorrhiza	*Pisolithus* sp., *Boletus* sp., *Laccaria* sp., *Amanita* sp.
Micronutrient suppliers		
1.	Silicate and zinc solubilizers	*Bacillus*, *Acinetobacter*, *Burkholderia*
2.	Iron sequestering	*Pseudomonas*, *Azotobacter*, *Rhodococcus*, *Mycobacteria*, *Rhizobia*, *Bacillus*, *Burkholderia*, *Arthrobacter*, *Actinobacteria*
3.	Potassium solubilizers	*Bacillus mucilaginosus*, *Azotobacter chroococcum*, *Aspergillus awamori*

Adapted from Reddy and Saravanan (2013)

inoculation method, where they proliferate and contribute in nutrient recycling process via nitrogen fixation, phosphate and potassium solubilization or mineralization. In addition, microbes are present in biofertilizers, release plant growth-promoting substances, produce antibiotics and degrade organic matter in the soil, which enhance crop productivity. Different types of biofertilizers like N_2 fixer, phosphate-solubilizing microorganisms and zinc- and potassium-solubilizing bacteria are reported to increase crop production, productivity and soil fertility (Kumar 2018). Different groups of microorganisms which act as biofertilizers are presented in Table 5.1.

Nitrogen-Fixing Microorganisms

Microorganisms fix atmospheric nitrogen in two different ways: (1) symbiotic fixation and (2) free living. Different types of microbes have been discovered to perform nitrogen-fixing ability. In symbiotic association, *Rhizobium* are well documented that can fix atmospheric nitrogen into soluble form in host plant root nodules. They can fix 50–300 kg N/ha per year with legumes only. Most of the rhizobia maintain obligate host relationship. For instance, *Bradyrhizobium japonicum* is associated with soybean as well as *Mesorhizobium ciceri* for chickpea, and many more interactions exist like this (Vaishnav et al. 2017a, b). In addition,

some non-symbiotic nitrogen free-living nitrogen-fixing organism like *Azotobacter* can fix 15–20 kg N/ha per year. These bacteria are also reported to increase germination, vigour in young plants and biocontrol activity against many plant pathogens leading to improved crop yields. *Azotobacter* spp. are documented in rhizospheric soil of different crop plants like rice (*Oryza sativa* L.), maize (*Zea mays* L.), sugarcane (*Saccharum officinarum* L.), bajra (*Pennisetum glaucum* L.), vegetables and plantation crops (Jnawali et al. 2015). The most common species which is found in arable soil is *A. chroococcum* (Wang et al. 2018). *Azospirillum* is also an associative nitrogen-fixing microorganism beneficial for nonleguminous plant which can fix about 20–40 kg/ha and also has the ability to produce various plant growth-promoting substances (Steenhoudt and Vanderleyden 2000). In cereals, *Herbaspirillum* are reported for nitrogen fixation, even as endophytes in apoplastic or intracellular locations. The genus *Herbaspirillum* was first reported in association with the roots of rice (*Oryza sativa*), maize (*Zea mays*) and sorghum (*Sorghum bicolor*). Later, a sugarcane pathogen *Pseudomonas rubrisubalbicans* was reclassified as *Herbaspirillum rubrisubalbicans.* Some varieties of sugarcane exhibit a nitrogen contribution of 40–60% from biological nitrogen fixation. For other crops, like sorghum and rice, a biological fixed nitrogen input of 20–30% was shown (Carvalho et al. 2014).

Blue-green algae (cyanobacteria) are phototropic prokaryotic organisms having ability to fix nitrogen through both symbiotic and asymbiotic associations. Anabaena blue-green algae have a symbiotic association with *Azolla* (a fern) in submerged paddy cultivation. This symbiotic relationship converts insoluble phosphate into soluble forms and fixes N_2 up to 2–30 kg/ha in the presence of bright sunlight. Examples of cyanobacteria are *Anabaena*, *Nostoc*, *Plectonema*, etc. Usually, the inoculums for field application contain two or more genera, since they are superior to single-strain inoculation (Berman-Frank et al. 2003). Use of *Azolla* as biofertilizer for rice crop is more popular in Vietnam rather than in India due to its unsuitability for rainfed rice-growing region (required proper and adequate water supply), low/high temperature tolerance and high susceptibility towards insects and diseases.

In fungi, *Trichoderma* species plays an important role in the decomposition process of organic matter that increases the nutrient status of soil and also enhances the nutrient availability to the crops. The secondary metabolites of *Trichoderma* have multifarious applications in agroindustry (Ram et al. 2016; Singh et al. 2012). Seed priming and seed treatment with *Trichoderma* species can increase nitrogen use efficiency (NUE) in plants and reduce the need of nitrogen fertilizers up to 30–50% (Singh 2014; Zhang et al. 2018). In addition, mycorrhizal fungi create a mycelial-root interaction network that expands the absorbing area and helps in assimilation of sparingly available nutrient sources. Mycorrhizal infection can provide host plants with access to N sources which are normally unavailable to non-mycorrhizal roots (Rillig et al. 2016).

Phosphate-Mineralizing and Phosphate-Solubilizing Microorganisms
Most of the phosphorus amount present in the soil is unavailable to plants. In acidic soil, phosphorus are in fixed form that occurs in both organic and inorganic forms. Organic phosphorus contributes 70–80% of total fixed phosphorus present in the soil. Phosphate-solubilizing bacteria (PSB) solubilize insoluble phosphorus and make available to the plant. PSB release organic acids in the soil that reduce pH and release phosphate from their bound forms. Some reports are also available in which phosphorus could be converted into soluble form without organic acid secretion. For example, *Trichoderma harzianum* T-22 showed P-solubilizing ability (rock P) without organic acid production (Khan et al. 2014). Datta et al. (2015) reported reduction in super phosphate amount used as well as increased yield upon PSB inoculation in plants. Fungal-solubilizing activity is also useful in the management of phytopathogens. Most of the PSB belongs to *Flavobacterium*, *Rhizobium*, *Erwinia*, *Micrococcus*, *Achromobacter*, *Pseudomonas*, *Burkholderia*, *Bacillus*, *Aerobacter* and others. These microbes have the ability to solubilize multiple inorganic phosphate compounds, such as hydroxyapatite, tri-calcium phosphate, rock phosphate and dicalcium phosphate. Some mycorrhiza are also reported to mineralize and solubilize the fixed form of phosphorus that is easily taken by plants resulting in increased yield (Sharma et al. 2013).

Zinc-Solubilizing Microorganisms
Zinc (Zn) is an essential micronutrient that plays a role in several metabolic reactions of plants. Plant is very much dependent upon nutrition of Zn throughout their life, as it is involved in more than 100 enzyme reactions such as auxin production, chlorophyll reactions, integrity of biological membranes and carbonic anhydrase and superoxide dismutase activities. It is also involved in grain quality by participating in nucleic acid, lipid and protein synthesis. Zn deficiency is closely related to nature of soil such as calcareous, sodic, saline, sandy and neutral soils (Vaishnav et al. 2016a, b). Zinc is present in the soil in different insoluble forms like hopeite, zincite, franklinite, smithsonite, zinkosite, etc. These insoluble forms have been tested for solubilization through PGPR. Rhizospheric bacteria solubilize bounded form of Zn and increase their uptake in plants. For example, fluorescent pseudomonads have been reported to enhance Zn uptake deficiency in wheat genotypes (Abaid-Ullah et al. 2015). Three PGPRs, namely, *Burkholderia* SG1(BC), *Acinetobacter* SG2 (AX) and *Acinetobacter* SG3 (AB), were found to produce gluconic acid in rhizosphere and increase Zn uptake in rice plants (Vaid et al. 2014). In addition, mycorrhizal association is also known to induce minerals uptake including Zn in pigeon pea, wheat, soybean and tomato plants (Srivastava et al. 2015).

Potassium-Solubilizing Microorganisms
Potassium (K) plays an essential role in protein and enzyme synthesis and photosynthesis process, as well as in plant immunity. Potassium present in available (water-soluble) and unavailable (orthoclase, micas and illite) forms in the soil. Potassium-solubilizing bacteria (KSB) are able to solubilize rock K or chelate silicon ions in the soil. KSB have importance in K uptake efficiency by plants, which

could help to reduce the dependency on costly chemical fertilizers (Ahmad et al. 2016). *Aspergillus awamori* has been used in composting and solubilization of mica and rock phosphate that release available form of K and used in crop production (Biswas and Narayanasamy 2006). *Azotobacter chroococcum*, *Bacillus mucilaginosus* and *Rhizobium* sp. have been reported to solubilize waste mica during hydroponic cultivation of wheat and maize (Singh et al. 2010).

Iron Sequestering Microorganisms

Iron plays a role as a cofactor in various enzymatic processes in plants. Although abundant in the environment, iron is not readily available to plants and microorganism. Its solubility is reduced in alkaline soil (Vaishnav et al. 2016a, b). Soil microorganisms have the ability to scavenge iron from unusable sources through different mechanisms like reduction of ferric into ferrous ion, use of ferritin (storage form of iron) and enzymatic degradation of iron complexes. However, among these mechanisms, siderophore production has been best studied in microbes. Siderophores are water-soluble, low-molecular-weight compounds that show strong affinity for iron binding. These compounds are important attribute of PGPRs for plant growth promotion and phytopathogen protection. Siderophores can be directly taken up by plant roots for iron uptake (Khan et al. 2017). Siderophore-producing bacteria have been found to associate with rice paddy soils (Loaces et al. 2011). In addition, a variety of siderophores have been produced by fungi (Winkelmann 2007). The nature of siderophores depends on their skeleton compounds like peptide, lipopeptide, di- and tri-amino alkane, citric acid, etc. Most studied siderophore-producing genera are *Pseudomonas*, *Azotobacter*, *Rhodococcus*, *Mycobacteria*, *Rhizobia*, *Bacillus*, *Burkholderia*, *Actinomycetes*, *Arthrobacter* and others.

Mycorrhizal Association

Arbuscular mycorrhizal associations have been reported with almost 80% of plant families that are involved in nutrient cycling. In addition, the role of AMF is also documented for enhancing plants tolerance against various biotic and abiotic stresses (Lone et al. 2017). The most studied order of AMF is *Glomales* including *Glomaceae* family together clustered with the *Acaulosporaceae* and *Gigasporaceae* in a monophyletic clade. Two clades, namely, *Archaeospora* and *Paraglomus*, are separated from *Glomaceae* (Schwarzott et al. 2001). Two different types of mycorrhizal associations are ectomycorrhiza and endomycorrhiza that have different structural as well as physiological bearings with the host plant. Mycorrhizal symbioses alter both the physical and chemical characteristics of the rhizosphere. AMF association is involved in nutrient cycling via glomalin accumulation (Ahanger et al. 2014). Glomalin is a proteinaceous compound that induces aggregation and stability of soil particles. In the mutual association, fungal hyphae increase root area of plants that explore more soil volume and enhance nutrient uptake efficiency. The AMF association is mainly responsible for phosphorus and carbon uptake. The increased phosphorus nutrition in AMF-inoculated plants has a direct effect on antioxidant production, nitrogen metabolism, vacuolar membrane integrity and ion compartmentalization leading to plant growth. Hence, AMF association also reduces deleterious impact of the higher salt concentration in plants. AMF can maintain the

K^+/Na^+ ratio by increased uptake of potassium and prevent Na uptake, thus leading to better stress adaptation (Porcel et al. 2012).

Trichoderma spp.

It is well studied and documented that the formulation of *Trichoderma* spp. has been very often used during crop cultivation for higher plant growth and yield. *Trichoderma* spp. are able to well colonize with plant root due to their competent nature in rhizosphere. The better root colonization directly contributes into nutrient uptake. *Trichoderma* influences plant growth by several mechanisms like production of plant growth hormones, solubilization and increased uptake of nutrients and suppression of soil pathogens (Ram and Singh 2018). The secondary metabolites of *Trichoderma* spp. have multifarious applications in agriculture, pharmaceuticals, cosmetics, beverage and allied sectors (Ram et al. 2016; Keswani et al. 2014). *T. harzianum* is documented to solubilize multiple mineral compounds such as MnO_2, metallic zinc and rock phosphate. Solubilization of different forms of phosphate has been reported by *Trichoderma* spp. in different crops leading to enhanced phosphorus availability to plants (Li et al. 2015). *Trichoderma* sp. is also documented for enhancement of potassium uptake in leaves and grains of chickpea (Bidyarani et al. 2016). Another strain *T. asperellum* T34 was evaluated for its effect on the uptake of micronutrients by wheat (*Triticum aestivum* L.) grown in a calcareous medium. Plants inoculated with T34 strain exhibited increased Fe concentration in Fe-deficient media (de Santiago et al. 2011).

5.2.1.2 Biopesticides

Biopesticides have received much attention in recent times to control plant disease. The major commercialized biopesticides that have high market demand in India are *T. harzianum*, *T. viride*, *P. fluorescens* and *B. thuringiensis* and are regulated by the Central Insecticide Board (CIB), Faridabad, India. Till date, about 970 microbial-based biopesticides products are registered with CIBRC (http://cibrc.nic.in/bpr.doc) under Sections 9(3B) and 9(3) of the Insecticides Act (1968), Government of India (Fig. 5.2). CIBRC has registered 568 fungal-based products under Section 9(3B) provisional, namely, *T. harzianum* (55), *T. viride* (270), *Beauveria bassiana* (95), *Metarhizium anisopliae* (37), *Verticillium chlamydosporium* (5) and *V. lecanii* (106), and bacterial products, *Pseudomonas fluorescens* (157), *B. thuringiensis* (58), *B. subtilis* (5) and *B. sphaericus* (3). Further under section 9(3), 68 products are registered by various companies.

Bacillus Thuringiensis

The most widely used biopesticide in the world is *B. thuringiensis* (Bt), which is an insect pathogenic gram-positive facultative-aerobic soil bacterium. This bacterium produces an insecticidal protein known as δ-endotoxins or Cry proteins during sporulation. When consumed by susceptible larvae, this protein activates in high pH environment of the midgut and caused lysis of gut cells. The Bt diversity and their higher efficacy for most lepidopteran and coleopteran larvae make it popular worldwide and account for more than 60% of the market for biopesticide (Sanchis and Bourguet 2008). The sources of Bt formulations are mainly from their strains of the

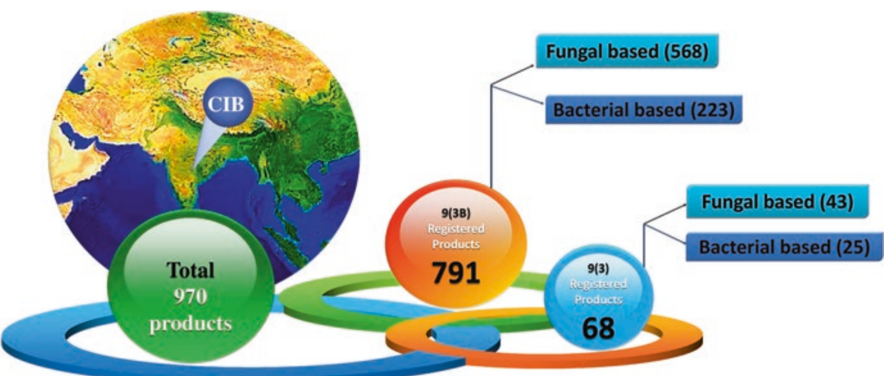

Fig. 5.2 Commercially registered biopesticides with CIBRC. (Source: www.cibrc.nic.in/G_biopesticides.doc)

subspecies *kurstaki*, *galeriae* and *dendrolimus*. Bt sprays are used to control caterpillar pests on vegetables and fruit, larvae of the gypsy moth, European corn borer and *Ostrinia nubilalis*. Bt formulations have also been found effective on various crops such as soybean, cotton and maize, where resistance to synthetic chemical insecticides is a problem. The toxins of Bt are also genetically engineered in several crop plants to make them resistant against insect attack (Chandler et al. 2011).

Entomopathogens

Other bioinsecticides are developed based on entomopathogenic baculoviruses and fungi. Baculoviruses are host target specific particularly affect to lepidopterous pests. For example, *Cydia pomonella* granulovirus (CpGV) is majorly used as most effective biopesticide against codling moth on apple crop in the United States of America (Chandler et al. 2011). In addition, nucleopolyhedro virus has been used on 35% soybean crop against caterpillar *Anticarsia gemmatalis* in Brazil (Moscardi 1999). The entomopathogenic fungi products are majorly based on *B. bassiana* and *Metarhizium anisopliae*. These fungi penetrate the host cuticle tissues and utilize nutrients present in the haemocoel and produce toxins to kill the insect. *B. bassiana*-based insecticide 'Boverin' has been found to suppress *Cydia pomonella* L. and reduced doses of trichlorophon, chemical insecticide (Ferron 1971). However, more desirable and faster results of *B. bassiana* spray have been reported along with low addition of imidacloprid (Ambethgar 2009). The *M. anisopliae*-based biopesticides have been used against spittle bugs on large production of sugarcane in Brazil (Li et al. 2010). The Food and Agriculture Organization of the United Nations (FAO) recommended this fungus for the management of locust in year 2007. The *M. anisopliae* is also used against adult *Aedes aegypti* and *Aedes albopictus* mosquitoes (Scholte et al. 2007).

Other Microorganisms

Other biopesticides used against plant pathogens include *Trichoderma* spp., *Pseudomonas* spp. and *Mycorrhizae. Trichoderma* is relevant for dryland crops such

as groundnut, black gram, green gram and chickpea, which are susceptible to *Fusarium*, *Rhizoctonia*, *Pythium* and other soilborne pathogens. It secretes a wide range of secondary metabolites (volatile, non-volatile, diffusable) for the suppression of pathogen attack in plant environment (Waghunde et al. 2016). *Pseudomonas* spp. are the most studied bacteria due to their broad-spectrum antagonistic activity and their effectiveness in colonizing the rhizosphere. They synthesize a variety of bioactive molecules such as siderophores, 2,4-diacetylphloroglucinol (2,4-DAPG), pyrrolnitrin, rhamnolipids, pyoluteorin, 2,5-dialkylresorcinol, phenazines, gluconic acid, hydrogen cyanide (HCN), quinolones and lipopeptides for inhibiting growth of plant pathogens (Raaijmakers and Mazzola 2012). It has been observed that *P. fluorescens* have the ability to well adapt in soil and colonize plant root, which make it effective for more than one plant species from distinct pathogens (Couillerot et al. 2009). *Mycorrhizae* can cover plant roots by forming a mat known as a fungal mat. By forming fungal mat, it provides a physical barrier to the invading soil pathogen such as insects, nematodes, bacteria and fungi (Harrier and Watson 2004). In an indirect way, mycorrhiza enhances nutrient uptake ability of plant roots, which makes plants more strong to tolerate or resist disease-causing organisms (Ortas et al. 2017).

Nonpathogenic Organisms

The application of nonpathogenic organism to control soilborne pathogens is also gaining interest in the context of biostimulant. The nonpathogenic organisms suppress pathogens through competing with them for nutrients and infection sites for colonization. In addition, plants induced 'memory' defences against the nonpathogenic determinants that lead to faster and stronger induction of basal tolerance mechanisms upon exposure to a pathogen (Vaishnav et al. 2018a). Alabouvette (1999) first time reported about the potentiality of nonpathogenic *Fusarium oxysporum* and fluorescent *Pseudomonas* spp. to suppress Fusarium wilt disease. These organisms are responsible for competition for carbon and iron. It has been shown that nonpathogenic *Fusarium* strains Fo47 control growth of *Fusarium* wilt control in suppressive soils (Alabouvette et al. 1979). In addition, strain Fo47 has been found to manage cucumber disease caused by *Pythium ultimum* through mycoparasitism and antibiosis process (Benhamou et al. 2002).

5.2.2 Microbes for Management of Abiotic and Biotic Stresses

Biotic and abiotic stresses cause serious effects on plant growth and productivity. There is an increasing concern to develop efficient low-cost technologies for stress management in plants. In this context, microorganisms are cosmopolitan in nature, and they possess inherent metabolic capabilities to mitigate both stresses (Vaishnav et al. 2014). While plant-microbe interaction is a vital component of the ecosystem, the microbes are regarded as natural partners that induce systemic responses in plants which render them to survive under adverse environmental conditions. The interaction between plants and microbes involves complex mechanisms. Studies at physiological, biochemical and molecular level are paving path for understanding the complex processes which exhibit defensive responses by the microorganisms

(Gopalakrishnan et al. 2015). Soil microorganisms residing under adverse conditions can serve in agriculture for increasing and maintaining crop productivity. These microorganisms can undergo plant growth promotion and disease suppression through mechanisms such as hormone secretion, nutrient mobilization and induction of systemic responses (Fig. 5.3).

5.2.2.1 Microbes in Abiotic Stress Alleviation

Some microorganisms involved in mitigation of various abiotic stresses include salinity (*Azospirillum* sp., *Pseudomonas syringae*, *P. fluorescens*, *Bacillus*), drought (*Azospirillum* sp., *P. putida*, *Bacillus* sp.) and nutrient deficiency (*Pseudomonas alcaligenes*, *Bacillus polymyxa*). In addition to bacteria, some tolerant spp. of *Trichoderma* are also reported in amelioration of abiotic stresses. Some spp. of AMF such as *Glomus mosseae*, *G. etunicatum*, *G. intraradices*, *G. fasciculatum*, *G. macrocarpum*, *G. coronatum*, etc. aid in mitigation of abiotic stresses in various crops by increasing nutrient uptake and osmolyte accumulations. Microbial-mediated stress mitigation approach is more preferred over traditional strategies. Application of these microbes facilitates mitigation of stresses in agriculture, thus opening a new avenue for their diverse role.

It is an increasing concern of microbial-mediated abiotic stress alleviation in plants (Nadeem et al. 2014; Souza et al. 2015; Vaishnav et al. 2018a). Different genera of rhizospheric microbes are involved in plant growth promotion and alleviation of abiotic stresses, i.e. *Pseudomonas* (Sorty et al. 2016; Vaishnav et al. 2015, 2016a, b), *Bacillus* (Tiwari et al. 2011; Kumari et al. 2015), *Azotobacter* (Sahoo et al. 2014a, b), *Azospirillum* (Omar et al. 2009), *Rhizobium* (Sorty et al. 2016), *Burkholderia Pantoea* (Sorty et al. 2016), *Methylobacterium* (Meena et al. 2012), *Enterobacter* (Sorty et al. 2016), *Bradyrhizobium* (Swaine et al. 2007; Panlada et al. 2013), cyanobacteria (Singh et al. 2011) and *Trichoderma* (Ahmad et al. 2015). A list of microbes used in abiotic stress management has been represented in Table 5.2.

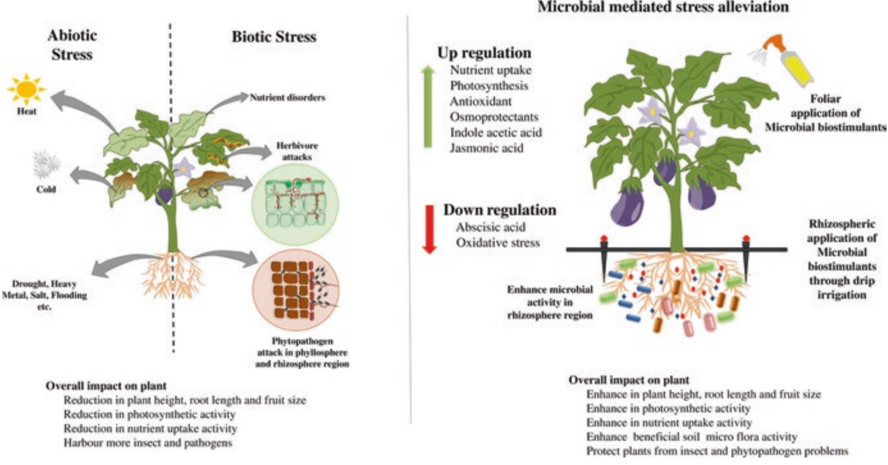

Fig. 5.3 Schematic representation of different stress responses in plants induced by biostimulants

Table 5.2 Microbes used in management of different abiotic stresses

S.N	Microbes	Plants	Stress	Tolerance mechanism	References
1.	*Pseudomonas polymyxa* and *Rhizobium tropici*	Common bean	Drought	Regulate stomatal conductance and hormonal balance in plant	Figueiredo et al. (2008)
2.	*P. mendocina* and *Glomus intraradices*	Lettuce	Drought	Induce antioxidant machinery in plant	Kohler et al. (2008)
3.	*Variovorax paradoxus*	Pea	Drought	Reduce ethylene level in plant	Dodd et al. (2005)
4.	*Rhizobium* spp.	Sunflower	Drought	Bacterial EPS induces soil aggregation that enhances survival of plant	Alami et al. (2000)
5.	*Pseudomonas* sp.	Pea	Drought	Bacterial ACC deaminase reduces deleterious ethylene level in plant	Arshad et al. (2008)
6.	*Rhizobium* spp.	Wheat	Drought	Bacterial inoculation improved water source and sink relation	Creus et al. (2004)
7.	*Paraphaeosphaeria quadriseptata*	*Arabidopsis*	Drought	Upregulation of heat shock proteins in plant	McLellan et al. (2007)
8.	AM fungi	Sorghum	Drought	AM fungus improves water relations in plant	Cho et al. (2006)
9.	*Bacillus licheniformis* strain K11	Capsicum	Drought	Bacterial inoculation induces stress-related genes and proteins in plants	Lim and Kim (2013)
10.	*Bacillus thuringiensis* AZP2	Wheat	Drought	Bacterial-mediated VOCs promote plant growth and tolerance	Timmusk et al. (2014)
11.	*Burkholderia phytofirmans and Enterobacter* sp. FD17	Maize	Drought	Bacterial inoculation affects plant physiology including photosynthesis	Naveed et al. (2014)

(continued)

Table 5.2 (continued)

S.N	Microbes	Plants	Stress	Tolerance mechanism	References
12.	*Pseudomonas chlororaphis* O6	*Arabidopsis*	Drought	Volatile compound 2R,3R-butanediol induces plant growth and tolerance	Cho et al. (2008)
13.	*Bacillus cereus* AR156, *B. subtilis* SM21 and *Serratia* sp. XY21	Cucumber	Drought	Bacterial inoculation induces accumulation of osmolytes and antioxidants	Wang et al. (2012)
14.	*Pseudomonas* and *Enterobacter*	Maize	Salinity	Decrease negative effect of ethylene and increase nutrient content in plants	Nadeem et al. (2009)
15.	*Rhizobium* and *Pseudomonas*	Mung bean	Salinity	Bacterial ACC deaminase activity enhances plant growth and metabolism in plant	Ahmad et al. (2011)
16.	*Brachybacterium saurashtrense* (JG- 06), *Brevibacterium casei* (JG-08) and *Haererohalobacter* (JG-11)	Groundnut	Salinity	Enhance phosphate and nitrogen content and reduce Na^+ content in plant	Shukla et al. (2012a, b)
17.	*Pseudomonas putida*	Canola and maize	Salinity	Bacterial ACC deaminase activity modulates plant protein expression	Cheng et al. (2012)
18.	*Pseudomonas pseudoalcaligenes* and *Bacillus pumilus*	Maize, rice GJ-17	Salinity	Bacterial inoculation induces antioxidants that lowers toxicity of ROS	Jha and Subramanian (2014)
19.	*Azospirillum* sp.	Wheat	Salinity	Regulate plant physiology	Nia et al. (2012)
20.	*Bacillus amyloliquefaciens* NBRISN13 (SN13)	Rice	Salinity	Modulate expression of stress-responsive gene expression	Nautiyal et al. (2000)

(continued)

Table 5.2 (continued)

S.N	Microbes	Plants	Stress	Tolerance mechanism	References
21.	*Pseudomonas* sp., *Serratia* sp.	Wheat	Salinity	Bacterial ACC deaminase activity enhances plant growth and metabolism in plant	Zahir et al. (2009)
22.	*Azospirillum* sp.	Lettuce seeds	Salinity	Induce antioxidant compounds in plant	Fasciglione et al. (2015)
23.	*Enterobacter* sp. JYX7 and *Klebsiella* sp. JYX10	Drooping knotweed	Cd, Zn and Pb toxicity	Bacterial PGP activities improved phytoremediation efficiency	Jing et al. (2014)
24.	*Bacillus thuringiensis* GDB-1	*Alnus*	Cu, Ni, As, Zn and Pb toxicity	Bacterial PGP activities improved phytoremediation efficiency	Babu et al. (2013)
25.	*Pseudomonas* spp. Lk9	European nightshade	Cu, Zn and Cd toxicity	Induce siderophore and organic acid production in soil that reduces toxicity	Chen et al. (2014)
26.	*Burkholderia phytofirmans*	Grapevine	Temperature	Bacterial inoculation induces osmolyte contents in plant	Barka et al. (2006)
27.	*Bacillus polymyxa*, *Mycobacterium phlei*, *Pseudomonas alcaligenes*	Maize	Nutrient deficiency	Increase N, P and K content in plant	Egamberdiyeva (2007)
28.	*Pseudomonas aeruginosa*	Wheat	Zn toxicity	Increase nutrient content in plant leading to high biomass and protein content	Islam et al. (2014)
29.	*Photobacterium* spp.	Common reed	Hg toxicity	Bacterial inoculation induces mercury reductase activity in plant	Mathew et al. (2015)
30.	*Bacillus subtilis*, *Bacillus megaterium*, *Bacillus* sp.	Rice	Fe toxicity	Bacterial siderophore reduces toxic concentrations of Fe	Asch and Padham (2005) and Terre et al. (2007)

Mechanism of Action of Biostimulants in Conferring Resistance Against Abiotic Stresses

1. ACC deaminase.
2. Production of volatile compounds.
3. Production of phytohormones.
4. Production of exopolysaccharides.

Aminocyclopropane-1-Carboxylase (ACC) Deaminase
The enzyme ACC deaminase was first characterized by Honma and Shimomura (1978) and has been reported to be involved in plant growth promotion (Glick et al. 1998). The ACC deaminase lowers the ACC levels, which acts as a precursor of ethylene. Thus, the bacterial endophytes producing ACC deaminase serve as excellent plant growth promoters by reducing ethylene production. It has been found that most of the ACC deaminase-containing PGPR can effectively protect against growth inhibition by various biotic and abiotic factors (Kumari et al. 2016a). ACC deaminase activity is one of the key enzymes involved in providing abiotic stress tolerance in plants (Kumari et al. 2016b. It has been found out that the mutants of bacterium *Burkholderia phytofirmans* PsJN lacking ACC deaminase activity were unable to promote root elongation of canola seedling which the nonmutants can do effectively (Sun et al. 2009).

The ACC deaminase activity of *Achromobacter piechaudii* was found to induce tolerance and promote growth in tomato and pepper plants against water deficit. Ethylene production was found to be lower in inoculated plants compared to non-inoculated controls, with enhanced recovery to water stress (Mayak et al. 2004b). Co-inoculation of two ACC deaminase-producing bacterial isolates *Bacillus* 23-B and *Pseudomonas* 6-P was studied on *Cicer arietinum* for mitigation of water stress. Inoculated plants exhibited improved germination, root and shoot length and fresh weight as compared to uninoculated plants. In another study, a mutant strain of ACC deaminase-producing bacterium *P. simiae* AU-M4 provides better tolerance to mung bean plants under drought stress as compared to wild-type strain. Plants inoculated with *P. simiae* AU-M4 exhibited lower ethylene level under stress condition (Kumari et al. 2016b).

The plant growth-promoting effects of *Pseudomonas fluorescens* TDK1 possessing ACC deaminase were more prominent in groundnut grown under saline field conditions (Saravanakumar and Samiyappan 2007). Similarly, transgenic canola possessing a bacterial ACC deaminase gene was more tolerant to elevated levels of salt than non-transformed control plants (Sergeeva et al. 2006). Although the actual mechanism is still in dark, it has been proposed that higher potassium and phosphorus uptake along with ACC deaminase activity plays a key role in plant growth promotion (Mayak et al. 2004a). Other studies reported inoculation of ACC deaminase-producing bacteria in salt-stressed maize plants induced higher K^+/Na^+ ratios in combination with elevated chlorophyll, relative water and low proline contents (Nadeem et al. 2007). ACC deaminase-producing endophytes were also reported to enhance tomato plant growth under salinity stress (Ali et al. 2012; Karthikeyan et al. 2012; Vaishnav et al. 2018b). In addition, other reports are also available on the

ability of ACC deaminase-producing endophytes in amelioration of osmotic and metal stress in plants (Zhang et al. 2011).

Volatile Organic Compounds (VOCs)

Certain PGPRs are reported to produce VOCs which facilitate plant growth promotion. The VOC emission by microorganisms was first reported by Ryu et al. (2003a, b), and it has been regarded as an important characteristic of plant-microbe interactions (Vaishnav et al. 2017a, b). The phenomenon of VOC emission is demonstrated by a wide genera of microbes, although the nature of VOCs emitted may vary among inter- and intraspecies (Kanchiswamy et al. 2015; Vaishnav et al. 2017a, b). The microbial VOCs also impart disease resistance and abiotic stress tolerance in addition to plant growth promotion. Induction of volatile compounds takes place when plants are subjected to a range of various stress conditions. These VOCs act as signalling factor for carrying out local and systemic responses within the plant and the neighbouring ones (Sharifi and Ryu 2018).

Bacterial VOCs have been reported to induce salt tolerance in many plants. For example, Zhang et al. (2008a) studied *Bacillus* sp. GB03-mediated VOCs effect on *Arabidopsis* plant under salt stress. Results showed greater biomass production and less Na^+ accumulation compared to nonbacterial-inoculated plants. The VOC-induced stress tolerance was noticed in wild-type plants but was absent in *hkt1* null mutant, suggesting the pivotal role of HKT1 in conferring the salt stress tolerance triggered by VOC. *Arabidopsis* exposed to GB03 VOCs have found to accumulate high levels of choline and glycine betaine than the control (Zhang et al. 2010). Fe^{2+} uptake was enhanced in *Arabidopsis* plants treated with GB03 VOCs (Farag et al. 2006).

2,3-Butanediol is also an important component of VOCs of few PGPR strains, viz. *Pseudomonas chlororaphis* strain O6, which facilitate plant growth promotion and disease resistance (Naseem and Bano 2014). Other compounds in VOCs such as acetic acid-induced biofilm formation constituted with exopolysacccharides (Chen et al. 2015). Nitroguaiacol, benzothiazol and quinoline compounds in *P. simiae* VOCs have been reported to alleviate salt stress in soybean plants through overexpression of salt-responsive genes (Vaishnav et al. 2016a, b). In some studies, the uptake of sulphur (S) compounds in plants has been reported through VOCs released by soil inhabitants (Meldau et al. 2013). In blend of microbial VOCs (mVOCs), dimethyl disulphide (DMDS) is a commonly known S-containing compound, which has managed to withstand growth in plants under sulphur stress (Meldau et al. 2013; Kanchiswamy et al. 2015).

Phytohormones

Plant hormones have an important role in growth and development. Several PGPRs/PGPFs have the ability to synthesize phytohormones, namely, IAA, gibberellins, cytokinins, abscisic acid and ethylene, which help plants to tolerate stresses and enhance growth (Gupta et al. 2016). Plant species treated with IAA-producing bacteria facilitate plant growth through formation of lateral roots and roots hairs and thus aid in more water and nutrient uptake by the plants (Dimkpa et al. 2009;

Mantelin and Touraine 2004). Wheat seeds inoculated with *Azospirillum brasilense* Sp245 showed higher osmotic adjustment, water potential, increased water content and lower elasticity values under drought conditions. The grain yield loss to drought was 26.5% and 14.1% in control and *Azospirillum*-inoculated plants, respectively (Creus et al. 2005; Molina-Favero et al. 2008).

Inoculation of common bean (*Phaseolus vulgaris* L.) with *A. brasilense* Cd has led to increased root area and root length as compared to non-inoculated plants under drought stress (German et al. 2000). Similarly, *A. brasilense* inoculation in maize seedlings has resulted in improved absolute and relative water contents as compared to control plants under drought-challenged conditions (Vurukonda et al. 2016). *Platycladus orientalis* seedlings inoculated with *Bacillus subtilis* increased drought tolerance through augmenting ABA levels in shoots and regulating stomatal conductance (Liu et al. 2013). Likewise, *Arabidopsis* plants treated with *Phyllobacterium brassicacearum* strain STM196 augment osmotic stress tolerance by increasing ABA content and reducing leaf transpiration and in turn manage water loss (Bresson et al. 2013). Recently, Vaishnav and Choudhary (2018) reported that *P. simiae* AU inoculation in soybean plants induced IAA and ABA content and reduced ethylene level leading to induced drought tolerance. *Arabidopsis* plants treated with *A. brasilense* Sp245 had higher levels of ABA compared to control (Cohen et al. 2008).

Exopolysaccharides

Exopolysaccharide (EPS) secreted by PGPR has demonstrated tremendous potential in improving soil properties and plant productivity. The EPS production is a stress response mechanism in several microbes that induce under stress conditions (Roberson and Firestone 1992). EPS possess unique water holding and adhering properties which facilitate in plant growth promotion. EPS has the ability to chelate cations under alkaline condition and help to alleviate stress condition for plants. It also induces soil aggregation and stabilization, which induce water retention capacity of soil. In a study, EPS-producing bacteria increased root-adhering soil and maintained higher water potential around the roots, leading to protect seedlings from drought stress (Sandhya et al. 2009). Inoculation of *Pseudomonas putida* strain P45 sp. in sunflower seedlings increased root-adhering soil/root tissue ratio, plant biomass and survival under water stress. Cho et al. (2008) studied the bVOCs effect on *Arabidopsis thaliana* plants under drought stress. 2R,3R-Butanediol in bVOCs was involved in drought tolerance in *A. thaliana* plant. A decrease of Na^+ content in wheat seedlings was observed upon inoculation of EPS-producing bacteria under salt stress (Ashraf et al. 2004). Similarly, maize plants inoculated with EPS-producing bacterial strains *Proteus penneri* (Pp1), *Pseudomonas aeruginosa* (Pa2) and *Alcaligenes faecalis* (AF3) have resulted in increased plant biomass, soil moisture content and osmolyte content and higher root and shoot length under drought stress (Naseem and Bano 2014). Inoculation of *Rhizobium leguminosarum* (LR-30), *Rhizobium phaseoli* (MR-2) and *Mesorhizobium ciceri* (CR-30 and CR-39) in wheat enabled the host crop to withstand drought stress.

Inoculation of the bacterial consortium demonstrated higher plant growth and drought tolerance (Hussain et al. 2014).

5.2.2.2 Microbes Used as Bioprotector for Managing Biotic Stress in Plant

There is an outsized range of plant beneficial microbes in nature that move with the plant in phyllosphere and rhizosphere region and shield them from numerous biotic stresses, e.g. phytopathogen and herbivore attacks. Plant unleashes root exudates and different chemicals that attract both the helpful microbes and phytopathogens, e.g. fungi, bacteria, viruses, MLOs and other microorganisms that lead to huge crop losses (Ramegowda and Senthil-Kumar 2015). Generally, this biotic stress causes nutritionary imbalance, secretion irregulation and physiological disorders in the plant. The utilization of pesticides to beat the harmful result of biotic stress conjointly will increase the crop cultivation price and is not safe for the setting because of their risky effects. However, the utilization of agriculturally important microbes for this purpose will solve the environmental sustainability issue and conjointly decreases the crop cultivation price (Singh 2014). Various microbes that have biocontrol potential and nonpathogenic to plant increase phytohormones within plant may use as microbial biostimulant. So the use of these beneficial microbes has been considered as the sustainable substitute for replacing the toxic agrochemicals (Ram et al. 2018; Singh et al. 2016). The defence against pathogens is usually achieved through activation of the cellular component further as cellular burst, reinforcement of tissues and accumulation of secondary metabolites. The defence signalling molecules such as jasmonic acid (JA) and salicylic acid (SA) play a lead role in signal transduction and imparting defence (Jain et al. 2014). Co-inoculation of PGPR with mycorrhizae has been found to ameliorate the biotic stress as well as aid in plant growth promotion (Dohroo et al. 2013).

Mechanism Used by Beneficial Microbes for Biotic Stress Tolerance

In order to attain better growth and development, the interactions between plant and beneficial microbe in natural environment are crucial. Microbial consortium acts as a key tool in management of several dreadous phytopathogens (Ram and Singh 2017; Jain et al. 2013). They play a key role in nutrient mobilization and providing protection against wide range of phytopathogens (Shoebitz et al. 2009). They are also used for managing soilborne diseases to switch the hazardous chemical pesticides with maintaining the crop yield under biotic stress condition (Table 5.3). Plant-microbe interactions often release diverse types of elicitors and elicit various biochemical modifications in plants. These alterations result in disease resistance to plant for a substantial time period. Following mechanisms discussed below are used by PGPMs for biotic stress tolerance.

VOCs in Biotic Stress Alleviation

Volatile organic compounds (VOCs) of microbial origin as precursor molecules for plant defence have come back into limelight owing to recent molecular studies (Ryu et al. 2003a, b; Choudhary et al. 2008). There are many reports regarding the various

Table 5.3 Different groups of microbial siderophores

S.N.	Siderophore group	Examples	Organism	Structure (Source: PubChem)
1.	Hydroxamate	Ferrichrome	Fungi (*Aspergillus*, *Ustilago*, *Penicillium*)	
		Ferrioxamine B	Bacteria	S243 P322 R292 S294 S151

(continued)

Table 5.3 (continued)

S.N.	Siderophore group	Examples	Organism	Structure (Source: PubChem)
2.	Catecholate	Enterobactin	*Escherichia coli*, *Salmonella typhimurium*	
3.	Carboxylate	Rhizobactin	*Rhizobium meliloti*	
4.	Mixed	Pyoverdine	*Pseudomonas* spp.	

VOCs produced by plant growth-promoting microbes (PGPMs) which play a pivotal role in plant defence and eliciting the plant growth. These conjointly provide the new perspective to plant-PGPM interaction. The plants have the flexibility to increase the level of resistances towards the phytopathogen when they got exposure to biotic stimuli provided by various PGPMs (Kai et al. 2009). The interaction of PGPM with plant at rhizospheric zones elicits induced systemic resistance (ISR) in plants (Farag et al. 2006). There are more than a thousand types of VOC blends in which some are well known, e.g. dodecane, 3-hydroxy-2-butanone, 2,3-butanediol, 2-tridecanol, 2-tridecanone, tetramethylpyrazine and 2-undecanone (Audrain et al. 2015; Penuelas et al. 2014; Sharifi and Ryu 2018). Among them, the most studied bacterial-originated VOCs are 3-hydroxy-2-butanone and 2,3-butanediol; their role is also confirmed in ISR elicitation process (Ryu et al. 2003a, b). Fungi also emit a diverse range of VOC mixes; approximately more than 250 types of VOCs have been identified. This blend basically contains alcohols, thioalcohols, thioesters, simple hydrocarbons, phenols and other derivatives (Chiron and Michelot 2005; Korpi et al. 2009). Bacterial VOC mixture also contains ammonia, HCN and phenazine-1-carboxylic acid having antifungal properties (Choudhary et al. 2008; Kai et al. 2009). The association of PGPM VOCs with ISR is commonly observed in common bean, cucumber and carnation crop, and they are found effective to reduce the disease incidence, respectively, against halo blight (*Pseudomonas syringae* pv. *phaseolicola*), anthracnose (*Colletotrichum orbiculare*) and *Fusarium* wilt. These VOCs are also used for seed priming and protect plant against various soilborne pathogens (Duffy and Défago 1997; Schnider et al. 1995; Cronin et al. 1997; Raaijmakers and Weller 1998; Raaijmakers and Weller 2001). Foliar application of these VOCs also enhances the plant resistance level against phytopathogen by triggering the ISR pathway through various signal molecules, e.g. jasmonic acid, salicylic acid and ethylene (Pieterse et al. 2002; Choudhary and Johri 2009). Bacterial VOCs promote plant health by suppressing the sporulation, spore germination and proliferation of phytopathogens in plant system (Mackie and Wheatley 1999; Fernando et al. 2005; Vespermann et al. 2007; Liu et al. 2008). A study revealed that 26 genera of *Streptomyces* have the ability to produce VOCs which contain alcohols, ketones, terpenoids, alkenes and thiols (Scholler et al. 2002). A marine *Streptomyces* sp. has been reported which releases VOCs having antibiotic properties (Dickschat et al. 2005). Similarly *Myxococcus xanthus* releases 42 different types of volatile mixture (Dickschat et al. 2004). *Bacillus subtilis* volatile blend is found effective for promoting plant growth attributes, e.g. enhancing the photosynthetic ability, plant size and root structure during long duration of exposure (Zhang et al. 2008b; Xie et al. 2009; Gutierrez-Luna et al. 2010). The role of VOCs from rhizospheric bacteria is also seen in upregulation of auxin biosynthesis and elicits the production of important phytohormones, e.g. indole-3-acetic acid, cytokinins and gibberellins (Ryu et al. 2003a, b). *Bacillus nematocida* B16 release a blend of volatile organic compounds, viz. benzaldehyde and 2-heptanone, which are fatal to nematode larvae. Once the bacteria come into contact with nematodes, they enter through the intestine of nematodes and secrete proteolytic enzymes, leading to the death of nematodes (Niu et al. 2010). The VOCs of fungi, especially 8-carbon

alcohols and ketones, behave as infochemical or semiochemicals for insect attraction and deterrence (Ramoni et al. 2001; Ômura et al. 2002; Luntz 2003). The volatile, i.e. 1-ocen-3-ol, plays a major role in upregulation of genes associated with jasmonic acid and ethylene signalling and also suppresses the pathogenic activity of *B. cinerea* (Kishimoto et al. 2007). Some endophytic fungi, viz. *Muscodor albus*, *Oxyporuslate marginatus* and *Trichoderma* sp., release diverse types of VOC mixtures that are antibacterial as well as antifungal in nature and are found to be effective in suppressing the growth of various phytopathogenic bacteria and fungi (Strobel et al. 2001, Lee et al. 2009, Nieto-Jacobo et al. 2017; Quintana-Rodriguez et al. 2018). The VOCs of any bacterial and fungal origin have various ways to interact with plant and phytopathogens, i.e. as immune enhancers, signalling modulators or defence stimulator molecules.

Siderophores in Biotic Stress Alleviation

Siderophores are organic compounds with low molecular weight (approximately 2000 Da) which might be produced by microorganisms growing in low iron conditions (Schwyn and Neilands 1987). The essential feature of these compounds is that they act as Fe-binding chelators to chelate the ferric iron (Fe^{3+}) from various environments and make it accessible for microbial and plant cells (Neilands and Nakamura 1991). Siderophores have gained immense attention in current years attributable to their potential role and application in plant protection. Iron (Fe) is a vital component for the growth and development of nearly all living microorganisms that acts as a catalyst in oxygen metabolism, electron transfer and DNA and RNA building block formations (Aguado-Santacruz et al. 2012). In comparison to other elements, Fe is likewise more vital for the phytopathogens because of their vital role in virulence, e.g. biofilm formation, sporulation and development (Simoes et al. 2007). Owing to the low bioavailability of Fe in the environment, it is being fastly utilized by bioinoculants (Weinberg 2004; Beneduzi et al. 2012). In this context, bioinoculants which have the ability to produce a large amount of siderophore can be applied into soil to compete with phytopathogens for Fe and suppress their growth. The nature of major bacterial siderophore is catecholates, while in fungal siderophore mixed hydroxamates are more common (Cornelis 2010; Renshaw et al. 2002; Winkelmann 2007).

The role of siderophores in plant growth promotion, enhanced nutrient uptake and protection from various biotic stresses has been well documented for a wide range of plant species. Rungin et al. (2012) reported that the use of siderophore of *Streptomyces* spp. has been effective to increase the growth promotion activities in rice and tomato crop. The role of *Pseudomonas putida* in rice for increasing nutrient uptake and enhancement of Fe content is reported (Sharma et al. 2013). Microorganisms use different paths for utilizing the siderophore-quenched iron. In fungi, four different types of mechanisms are found to transport these siderophores for utilization. Gram-positive and gram-negative bacteria both use different strategies to transport the quenched iron (Krewulak and Vogel 2008; Fukushima et al. 2013). During the low iron availability, the microbial siderophores help plants to provide iron for their growth (Crowley 2006; Masalha et al. 2000). The role of

microbial siderophores in the management of phytopathogens also has been reported; they can be used as safer eco-friendly alternative to hazardous agrochemicals. Several types of siderophores (Table 5.4) have been reported from different bacterial and fungal genera (Park et al. 2005a, b; Temirov et al. 2003; Wilson et al. 2006; Gamalero and Glick 2011; Schenk et al. 2012). Recent studies on siderophore-producing fungi (e.g. *Trichoderma* spp., *Aspergillus niger* and *Penicillium citrinum*) revealed that they are also used as bioprotectant separately instead of using the fungi as bioinoculants.

Antimicrobial Compounds in Biotic Stress Alleviation
AIMs produce a large variety of antibacterial and antifungal compounds which act on different types of phytopathogens and suppress their growth in plant system (Sturz et al. 2000; Strobel 2003). The use of these antimicrobial compounds has increased in the last three decades due to high toxic impact of synthetic agrochemicals in environment. Numerous fungal and bacterial genera have been recognized which produce a wide range of antimicrobial compounds. Gliotoxin was first reported as an antagonistic compound to *Rhizoctonia solani* which causes foot root in potato and tomato, isolated from fungus *Gliocladium fimbriatum* (Weindling 1932). After that a wide range of compounds were identified, viz. penicillin, streptomycin, aureofungin, cycloheximide, griseofulvin, Ohyamycin and blasticidin, having antimicrobial properties. There are studies that reported the involvement of iturin A, agrocin 84, phenazine and 2,4-diacetyl in the suppression of phytopathogens, namely, *Botrytis cinerea*, *Agrobacterium tumefaciens*, *Gaeumannomyces graminis* var. *tritici* and *Pythium* spp. Strobel et al. (1997) reported an antifungal compound called leucinostain A, from *Acremonium* sp. showing antagonism against various soilborne pathogens. Table 5.4 illustrates a list of several antimicrobial compounds isolated from various endophytic microbes.

5.3 Conclusion

Biostimulants play a crucial role in crop production through amelioration of abiotic and biotic stresses. These stresses act as major hindrances for quality crop production, food safety and global food security. One of the key alternative solutions to these plant stresses is to design and develop some microbial tools and techniques for efficient plant-microbe interaction. These microbes aid in plant growth by regulating plant hormones, siderophore production, improving nutrition and augmentation of the antioxidant mechanism. Microbial biostimulants are gaining widespread publicity in recent years due to their eco-friendly and target-specific nature. In order to obtain optimum application rates, plant-biostimulant specificities and techniques should be standardized which may deliver highest impact on sustainable crop production. The global market for biostimulants has been projected to attain 2241 million dollars by 2020 with an annual growth rate of 12.5% in the last 5 years (Anonymous 2013). However, an organized methodology based on omics approach has been anticipated to identify and develop novel microbial

Table 5.4 Representative list of antimicrobial compound isolated from various microbes

S.N.	Antimicrobial compounds	Isolated organism	Target organism	References
1.	Cryptocin	*Cryptosporiopsis quercina*	*Magnaporthe oryzae*	Li et al. (2000)
2.	Cerebrosides	*Fusarium* sp.	*Bacillus subtilis*, *Escherichia. coli*, *Pseudomonas fluorescens*	Shu et al. (2004)
3.	Phomodione	*Phoma pinodella*	*Pythium ultimatum*, *Sclerotinia sclerotiorum*, *Rhizoctonia solani*	Hoffman et al. (2008)
4.	Pyrrocindine A and B	*Acremonium zeae*	*Aspergillus flavus*, *Fusarium verticillioides*	Donald et al. (2005)
5.	6-Isoprenylindole-3-carboxylic acid	*Colletotrichum* sp.	*Bacillus subtilis*, *Pseudomonas fluorescens*, *Phytophthora capsici*, *Rhizoctonia cerealis*	Lu et al. (2000)
6.	Enniatins A1, B, B3	*Fusarium proliferatum*	*Botrytis cinerea*	Ji et al. (2005)
7.	3β,5α-Dihydroxy-6β-acetoxy-ergosta-7,22-diene	*Colletotrichum* spp.	*Bacillus subtilis*, *Pseudomonas* spp., *Aspergillus niger*	Lu et al. (2000)
8.	Isopestacin and pestacin	*Pestalotiopsis microspora*	*Pythium ultimum*	Harper et al. (2003)
9.	Annulene	*Gliocladium* spp.	*Pythium ultimum*, *Verticillium dahliae*	Stinson et al. (2003)
10.	Rubrofusarin B	*Aspergillus Niger*	*E. coli*, *Bacillus subtilis*, *P. fluorescens*	Song et al. (2004)
11.	Griseofulvin	*Xylaria* spp.	*Magnaporthe oryzae*, *Phytophthora infestans*, *Botrytis cinerea*, *Blumeria graminis* f.sp. *hordei*	Park et al. (2005a, b)

biostimulants and elucidate their mode of action. We have already reached a crucial stage where there is a need to derive and identify novel microbial biostimulants which are produced under stressed environmental conditions. The established results reveal that development made in biostimulants till date has shown a promising future for nutrient fortification and soil disease management. However, a more detailed study using metabolomics-based approach may help to reveal hidden keys that play crucial roles in plant-microbe interactions for agricultural benefits.

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6 Ectomycorrhizal Diversity and Tree Sustainability

Shikha Khullar and M. Sudhakara Reddy

Abstract

Ectomycorrhizal (ECM) association of fungi with plants involves diverse category of fungi. They form a mutualistic association with the host plant, nourishing them with minerals and protecting them from various biotic and abiotic stresses. Their long thin hyphae fetch water and minerals from the deepest core of soil and transport them to the plants. In exchange, ECM fungi are rewarded with photosynthates and carbohydrates. They also protect the host plant from drought, salinity, heavy metals, pests and pathogens and extreme environments, thus enhancing their growth and development and helping them to sustain under diverse conditions. Colonization with ECM fungi modulates various cellular, physiological and molecular processes in host plant, thus protecting them under extreme environments. ECM fungi play a significant role in protecting the forest ecology by connecting different trees through a dense network of hyphae forming a wood-wide web of common mycorrhizal networks. However, each mycorrhizal fungus responds differently under different stress conditions through diverse mechanisms. The current study provides deep insight into different mechanisms used by different ECM fungi for facilitating host tree sustainability.

Keywords

Ectomycorrhizal fungi · Mineral nutrition · Tree sustainability · Salinity · Metal tolerance · Metallothioneins · Drought tolerance · Diversity

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6.1 Introduction

Mycorrhizal fungi are mutualistic root symbionts with heterogeneous fungal taxa that thrive in the rhizospheric soil. In this association, both plant and fungi assist each other with various nutritional and non-nutritional benefits. Mycorrhizas form a dense filamentous network that draws water and nutrients from the deeper soil and delivers to the plant roots, thus accelerating plant growth and root development. It is estimated that every kilometre of soil contains at least 200 km of fungal strands, accessing the smallest pore of the soil (Bonneville et al. 2009). Apart from providing nutrition, mycorrhizal fungi also protect the plant from drought and salinity, pests and pathogens, heavy metal toxicity and extreme environments (Smith and Smith 2015). In exchange to all these benefits, the mycorrhizas are awarded with photosynthates or carbohydrates like glucose by the plants. The fungi utilize these carbohydrates for their growth and synthesis of glycoprotein glomalin which is released into the soil that improves the soil structure and organic content (Wu et al. 2014). In the soil, the mycorrhizal hyphae form an extensive network of wood-wide web, which connect all surrounding plant communities promoting horizontal nutrition transfer (Harrison 2005). They are the source of carbon to many achlorophyllous heterotrophic plants. Alpines and boreal zones, tropical forests, grasslands and croplands are the most commonly mycorrhizal colonized areas (Selosse and Roy 2009).

These mycorrhizal fungi are more than 450 million years old. About 80% of all land plants with more than 92% plant families are mycorrhizal (Wang and Qiu 2006). They are most prevalent symbionts involving about 6000 fungal species in the *Glomeromycotina*, *Ascomycotina* and *Basidiomycotina* and 240,000 plant species (Bonfante 2003). Mycorrhizas are cosmopolitan and abundant even in the highly degraded areas. They are classified into five groups based on their characteristic interaction with the host cells. Among the five associations, arbuscular mycorrhizas (AM) and ectomycorrhizas (ECMs) are the two most abundant associations. Orchid, ericoid and arbutoid mycorrhizas are confined to the genera within the Orchidaceae, Ericaceae and Arbutoideae families, respectively (Brundrett 2017). Arbuscular, orchid, ericoid and arbutoid mycorrhizas establish an intracellular symbiosis by penetrating their hyphae into the root cells, whereas the ectomycorrhizal hyphae remain extracellular (Bonfante and Genre 2010). Till date, mycorrhizas have garnered vast attention owing to the new genetic, cellular and molecular techniques along with the genome sequencing of various mycorrhizal plants and fungi (Martin et al. 2008).

6.2 Ectomycorrhizas

Ectomycorrhizal fungi is a symbiotic association of fungi to the plant roots, where the fungi ensheath its hyphae around the root tip forming the thick hyphal mantle of nearly 40 μm. Inside the mantle, the hyphae penetrate into the cell wall and grow in between epidermal cells and cortical cells. They never penetrate inside the cell

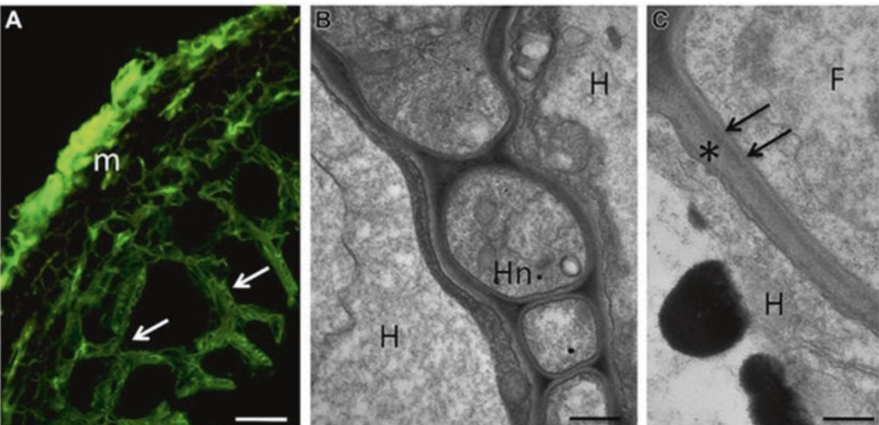

Fig. 6.1 The hyphae of ECM fungi colonizing epidermal and cortical cell layer. (**a**) The confocal micrograph of ECM *Tuber melanosporum* colonizing hazelnut, indicating the mantle (m) formed by the dense hyphae and Hartig net (arrows) surrounding epidermal and cortical cells. (**b**) Hartig net (Hn) developed in a fully truffle developed mycorrhiza. (**c**) Magnification of the contact zone between plant (*) and fungal cell wall (arrows). *F* fungus, *H* host cell (Balestrini and Bonfante 2014)

lumen of roots, hence forming a Hartig net (Fig. 6.1). This Hartig net is the region of juxtaposition where exchange of water, nutrients and other components between both fungi and the plant roots takes place (Bonfante and Genre 2010; Balestrini and Bonfante 2014; Henke et al. 2015).

On the other end outside the fungal mantle, hyphae extend into the soil called extraradical mycelium and act like an extension to the plant roots so as to have access to the nutrients from deeper parts in soil. In pine seedlings, extraradical mycelium of *Pisolithus tinctorius* contributes 99% of the nutrient-absorbing surface of roots (Rousseau et al. 1992). Thus, ectomycorrhizal fungi provide water, mineral and nutrients to the plant and are rewarded with the photosynthates or stored carbohydrates by the host plant (Fig. 6.2) [Martin and Nehls 2009; Bonfante and Genre 2010]. These fungi have a dual lifestyle, form symbiosis with the plant roots and act as facultative saprotrophs in soil (Martin and Nehls 2009). At laboratory conditions they can be cultured without host (unlike AMF), but under field conditions they depend on their host for carbohydrates (Van Der Heijden et al. 2015).

Ectomycorrhizal fungi belong to the family of *Basidiomycota* (252 genera), *Ascomycota* (84 genera) and *Mucoromycota-Endogonales* (Van Der Heijden et al. 2015). More than 10,000 fungal species have been estimated to form ectomycorrhiza with the host plants (Finlay 2008). They can form a visible reproductive structure of epigeous mushroom and hypogeous truffles at the feet of trees they colonize. The most popular edible mushrooms 'Tuber' fungus, deadly caps destroying angels *Amanita*, also belong to ectomycorrhizas. Trees hosts for ectomycorrhizal fungi include families like Pinaceae, Fagaceae, Betulaceae, Dipterocarpaceae and Caesalpiniaceae, distributed in the sub-tropic, temperate and boreal forests (Smith

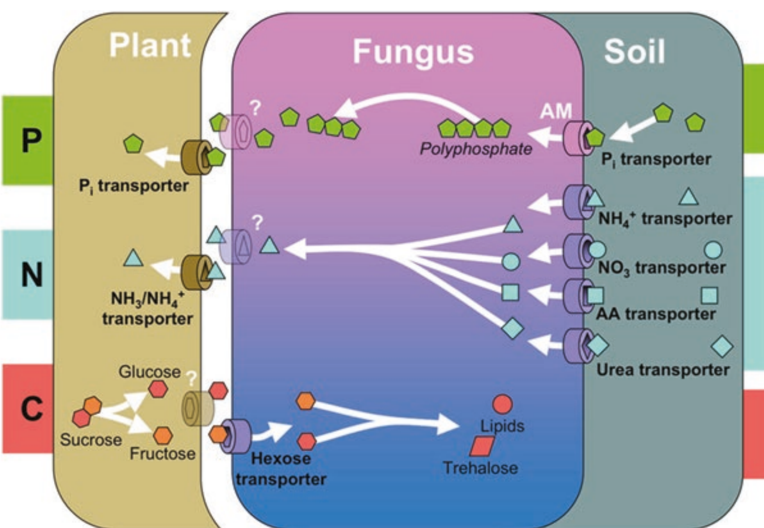

Fig. 6.2 Nutrient exchange mechanism between ECM fungi and their host plant (Bonfante and Genre 2010)

and Read 2010). Birch (*Betula*), dipterocarp (*Pakaraimaea*), beech (*Fagus*), willows (*Salix*), pine (*Pinus*), oaks (*Quercus*) and eucalypts (*Eucalyptus*) are the common host species for ectomycorrhizal symbiosis (Chen et al. 2015; Hrynkiewicz et al. 2015; Murata et al. 2015; Brearley et al. 2016; García-Guzmán et al. 2017; Horton et al. 2017; Kaiser et al. 2017).

It has been estimated that more than 50,000 plant species are involved in ectomycorrhizal association. Most of the ectomycorrhizas have colonized northern temperate forest soils, whereas arbuscular mycorrhizas are commonly found in tropical forests. *Agaricomycetes* were found to be the most dominant class of ectomycorrhizal fungi in the soil (Buée et al. 2009). Ascomycetes have been reported showing higher stress tolerance as compared to basidiomycetes. Many authors have observed the dominance of ascomycetes in heavily polluted areas, whereas basidiomycetes were observed in less polluted and control plots. The genera *Phialophora*, *Phialocephala* and *Leptodontidium* and many other ascomycetes have been reported to have adaptive metal tolerance (Colpaert et al. 2011). Similarly many basidiomycetes have also been reported to have complete defence mechanisms against heavy metals like *Laccaria bicolor* (Reddy et al. 2014), *Hebeloma cylindrosporum* (Ramesh et al. 2009), *Pisolithus albus* (Reddy et al. 2016), *Suillus bovinus* (Ruytinx et al. 2013), *Suillus luteus* (Nguyen et al. 2017), *Paxillus involutus* (Bellion et al. 2007), *Amanita strobiliformis* (Osobová et al. 2011), etc.

There has been a tremendous advancement in research concerning identification of ectomycorrhizal fungi and studying their ecological importance. Such studies have provided a deep insight into ectomycorrhizal diversity and their role in tree sustainability.

6.3 Ectomycorrhiza and Tree Sustainability

6.3.1 ECM and Tree Sustainability Under Drought

Drought is one of the most daunting factors limiting the plant growth in both dry and irrigated agriculture. The change in climatic conditions and increasing demand of water by municipal and residential consumption have worsened the soil water levels. This water deficiency affects various morphological and biochemical processes in plants. However, ECM fungi improve the water and nutrient uptake during drought conditions (Lehto and Zwiazek 2011). The colonization of drought-affected plants with ectomycorrhizal fungus can profoundly modulate the plant's response in improving its water availability and growth by increasing stomatal conductance and shoot water potential, enhancing photosynthesis, increasing hydraulic conductance and increasing aquaporin function (Lehto and Zwiazek 2011). When subjected to drought conditions, *Populus canescens* inoculated with *Paxillus involutus* showed higher predawn water potential and maintained almost full photosynthetic activities, whereas the non-mycorrhizal plants were severely affected by drought in hydrogel-amended soil (Beniwal et al. 2010). The *Populus canescens* plants showed higher stomatal conductance and nitrogen uptake when colonized with *Paxillus involutus* (Danielsen and Polle 2014). Switchgrass (*Panicum virgatum L.*) when inoculated with ectomycorrhizal fungus *Sebacina vermifera* showed enhancement in biomass and macronutrient content under drought conditions (Ghimire and Craven 2011). Even in *Pinus muricata* seedlings inoculated with *Rhizopogon* spp., the plant showed improved shoot biomass, photosynthesis and total leaf nitrogen at low moisture (13%). However both plant and ECM were severely affected when the moisture was reduced to 7% (Kennedy and Peay 2007).

There are several mechanisms by which the ECM fungi can promote drought tolerance. Different studies have reported different mechanisms. Firstly, the ECM fungi increase the surface area of root for absorption of water. Its extensive extraradical hyphae absorb water from the deeper soil and transport it to the host plant roots, resulting in higher root conductance and increased carbon assimilation under drought stress (Plamboeck et al. 2007; Sebastiana et al. 2018). Up-regulation in aquaporin (transporter facilitating water uptake) expression under drought conditions has been observed in many cases (Marjanović et al. 2005). Cork oak when inoculated with *Pisolithus tinctorius* resulted in improved plant height, shoot biomass, shoot basal diameter and root growth than their non-mycorrhizal plants under drought conditions (Sebastiana et al. 2018). Another indirect mechanism would be by facilitating the nutrient acquisition. The mineral nutrients improve the plants photosynthetic machinery facilitating quick recovery after stress. This has been reported in case of *Pinus tabulaeformis* seedlings and *Nothofagus dombeyi*, where the ECM fungal hyphae promoted the plants resistance to drought by facilitating N and P uptake (Wu et al. 1999; Alvarez et al. 2009). Another indirect mechanism involved is by suppressing the reactive oxygen species (ROS) generated during drought conditions by activating the plants antioxidant system (Porcel and Ruiz-Lozano 2004; Alvarez et al. 2009). Many other indirect mechanisms have also been

reported including altered carbohydrate assimilation by stomatal function mediated by change in growth regulators, increased sink strength and change in osmotic adjustments in ectomycorrhizal roots (Lehto and Zwiazek 2011). However a detailed mechanism on how ECM protects the host plant from drought stress is still unexplored.

6.3.2 ECM and Tree Sustainability in Saline Soils

Salt salinity is one of the major constraints limiting plant growth and ecosystem productivity. It is a major issue for both agriculture and forestry in nearly all climatic regions and on all populated continents. More than 6% of the world's total land area is affected by salinity, and the figures are increasing rapidly due to the intensive use of land, irrigation and clearing. Saline salt can be defined as the salt with electrolyte concentration more than 4 dS m^{-1} which is equivalent to 40 mM or higher concentration of sodium chloride (Munns and Tester 2008). High salt concentrations can alter the basic texture of soil, resulting in decreased soil porosity leading to reduced soil aeration and water conductance (Porcel et al. 2012). This creates a low water potential zone, making it difficult for plants to acquire water as well as nutrients. Thus, salinity stress usually overlaps drought and nutritional deficiencies. The higher salt concentrations in soil affect the plant growth, photosynthesis, protein synthesis and energy and lipid metabolism (Chen and Polle 2010). Symbiotic association of the plants with mycorrhiza has proven to be the most effective remedy to salt stress. The mycorrhizas are well known to protect the host plant from various biotic and abiotic stresses. However, the role of ectomycorrhizal fungi has not yet been explored adequately.

Mycorrhizal plants exposed to osmotic constraints have been reported to perform better than the non-mycorrhizal plants (Langenfeld-Heyser et al. 2007). The ectomycorrhizal symbiosis has been demonstrated to increase resistance to saline conditions in many plants including *Populus canescens*, *Populus tremuloides*, *Populus euphratica*, *Pinus tabulaeformis*, *Betula papyrifera*, *Coccoloba uvifera*, etc. (Bandou et al. 2006; Yi et al. 2008; Luo et al. 2011; Jie et al. 2011; Chen et al. 2014). The reduced Na^+ accumulation in ectomycorrhizal roots under salt stress has also been reported in *Picea mariana*, *Pinus banksiana* and *Picea glauca* when colonized with *Hebeloma crustuliniforme* (Muhsin and Zwiazek 2002; Nguyen et al. 2006) and in *Populus canescens* colonized with *P. involutus*. This is due to the fact that the mycelial hyphae exhibit steady Na^+ efflux under salt stress (Li et al. 2012). Although ECM colonization has been reported to reduce with increase in salt concentrations, the ECM dependency of the plant increased under saline conditions (Bandou et al. 2006). Sea grapes (*Coccoloba uvifera*) when colonized with *Scleroderma bermudense* under salt stress led to reduced Na and Cl uptake along with a concomitant increase in P, K and water absorption than the non-mycorrhizal plants (Bandou et al. 2006). Similarly, in case of *Populus canescens*, the colonization with *Paxillus involutus* increased root cell volume and nutrition uptake reducing the Na accumulation. The ECM roots showed higher accumulation of myoinositol, abscisic acid and

salicylic acid and decreased concentrations of jasmonic acid and auxin than the non-mycorrhizal roots. These observations are in contrast to that of arbuscular mycorrhiza, suggesting that ectomycorrhizal and arbuscular mycorrhizas follow different signaling pathways to influence the salt stress response to plants (Luo et al. 2009). Not only roots, the colonization of *Populus canescens* with *Paxillus involutus* also modulates the leaf physiology towards improving salt tolerance (Luo et al. 2011). The leaves of ECM plant displayed increased concentrations of phosphorus and potassium, stress metabolite γ-amino butyric acid, abscisic acid, glucose and fructose and decreased concentrations of galactose and unsaturated to saturated fatty acids than the non-mycorrhizal plants. The ECM-plant leaves under salinity showed alleviation in leaf chlorosis, improved water status and K^+ to Na^+ ratio (Langenfeld-Heyser et al. 2007; Luo et al. 2011).

It has been found that the K^+/Na^+ homeostasis in plants under salt stress has been influenced by the calcium (Ca^{2+}) enrichment in the fungal hyphae (Li et al. 2012;Chen et al. 2014). Under salt stress, Ca^{2+} is replaced by Na^+, and the released Ca^{2+} contributes to the amelioration of K^+/Na^+ ratio. The Ca^{2+} ions also inhibit KORCs and NSCCs (depolarization-activated) to reduce K^+ efflux in *Populus* (Sun et al. 2009; Li et al. 2012) and *Arabidopsis* (Shabala et al. 2005). Thus, we can say that salt stress induces Ca^{2+}/Na^+ exchange, resulting in availability of free Ca^{2+} from ectomycorrhizal roots, which favours the establishment of K^+/Na^+ homeostasis in plants (Li et al. 2012). This indicates that ectomycorrhizal symbiosis plays a significant role in tree sustainability under osmotic stress conditions.

6.3.3 ECM-Mediated Tree Sustainability Against Pests and Pathogens

Plant diseases are a serious risk to the global food security costing 10–16% loss of total crop harvest annually to pests and pathogens (Strange and Scott 2005; Oerke 2006). Fungi, oomycetes, bacteria, viruses, viroids, phytoplasmas, protozoa, insects, mites, weeds, nematodes and parasitic plants are among the most common plant pathogens. Although there are many methods to control plant diseases like using fungicides, bactericide, chemical pesticides, different irrigation practices, etc., they all cause notorious environmental and health consequences (Tilman et al. 2002). However, 'biocontrol' using mycorrhizal fungi has proven to be the most effective and efficient method of protecting the plant from pests and pathogens. Both ECM and AM have proven to protect the plant from various pests and pathogens. These fungi not only protect the plant from pathogens but also improve the plant health, growth and nutrition availability. Studies have reported numerous mechanisms by which the ectomycorrhizal fungi protect the host plant from different pests and pathogens (Ghorbanpour et al. 2018). The first mechanism that is widely reported is the barrier action of ECM fungi. ECM fungi penetrate into the root forming a thick mantle, which acts like a mechanical barrier against the penetration of various pathogens. *Castanea sativa* colonized with four different ECM fungi – *Laccaria laccata*, *Hebeloma sinapizans*, *H. crustuliniforme* and *Paxillus involutus* – showed

no sign of infection when inoculated with ink disease factors *Phytophthora cambivora* and *P. cinnamomi*, whereas the same seedlings without mycorrhization showed significant effect on leaf area, root and shoot weight (Branzanti et al. 1999; Blom et al. 2009). Apart from physical barrier, the ECM fungi also have an antagonistic property, where they chemically inhibit the pathogens. The ECM fungi *Suillus laricinus*, *Suillus tomentosus* and *Amanita vaginata* inhibited the growth of *Rhizoctonia solani* by synthesizing a hydrolytic enzyme chitinase (Tang et al. 2008). In a recent study, the antagonistic potential of eight ECM fungi including *Alnicola* sp., *Russula parazurea*, *Lycoperdon perlatum*, *Laccaria fraterna*, *Pisolithus albus*, *Suillus subluteus*, *Scleroderma citrinum* and *Suillus brevipes* was studied against various plant pathogenic fungi like *Alternaria solani*, *Lasiodiplodia theobromae*, *Botrytis* sp., *Fusarium oxysporum*, *Pythium* sp., *Rhizoctonia solani*, *Phytophthora* sp., *Sclerotium rolfsii* and *Subramaniospora vesiculosa.* All the eight ECM fungal isolates showed inhibitory effect to the selected plant pathogens. *Suillus brevipes* showed maximum inhibition of 60.31% producing maximum chitinase (111.6 μg/ml) followed by *S. subluteus* exhibiting 49.46% inhibition producing 101.7 μg/ml chitinase (Mohan et al. 2015). Similar observations have been made by Vaidya et al. (2005), where the ECM fungi *Pisolithus* and *Scleroderma* showed antifungal and antibacterial activity against plant pathogens *Pythium* sp., *Rhizoctonia solani*, *Fusarium* sp., *Agrobacterium tumefaciens*, *Pseudomonas solanacearum*, *Klebsiella* sp., *Staphylococcus aureus*, *Shigella dysenteriae* and *E. coli* (Vaidya et al. 2005). The ECM fungi *Pisolithus tinctorius* and *Pisolithus arhizus* have been reported to synthesize two antibiotic compounds *p*-hydroxybenzoylformic acid and (*R*)-(−)-*p*-hydroxymandelic acid which inhibited spore germination and caused hyphal lysis in significant number of phytopathogenic and dermatogenic fungi. Both the compounds were effective in inhibiting germination of conidia of *Truncatella hartigii* (Kope et al. 1991; Tsantrizos et al. 1991). *Paxillus involutus* also produced a fungitoxic compound while growing on *Pinus resinosa* roots, which inhibited 80% sporulation of *Fusarium oxysporum* (Duchesne et al. 1988).

Apart from the antagonistic properties, the ECM fungi alter the soil chemical properties in their close vicinity by lowering the soil pH and acidification, thus repressing the growth of pathogen such as *Cylindrocarpon* sp. and protecting the plant from various pathogens (Schelkle and Peterson 1997). Another indirect mechanism of protecting the host plant from pathogens includes competing with pathogens for niche and nutrition. The ECM fungi create a specific niche by colonizing the rhizosphere, thus depriving pathogens of space and nutrients. The ECM fungi create niche either by remaining in high population density or by forming symbiosis with different bacterial communities having antibiosis effect on plant pathogens (Frey-Klett et al. 2005). Schelkle and Peterson (1997) reported the presence of *Bacillus subtilis* in ECM rhizosphere as a biocontrol against *Fusarium oxysporum* and *Cylindrocarpon* sp.

The ECM fungi also protect the host plant from various insects and nematode herbivores. *Betula pubescens* seedlings colonized with ECM fungi *Paxillus involutus*, *Phialophora finlandia*, *Cenococcum geophilum* and *Thelephora terrestris* reduced the *Otiorhynchus* spp. larval root herbivory in birch forest and eroded sites

(Oddsdottir et al. 2010a, b). The effect of ECM fungi on herbivores may be due to the secretion of terpenes or polyphenolic compounds (Fitter and Garbaye 1994).

6.3.4 ECM and Tree Sustainability Under Heavy Metal Stress

Heavy metals contamination is one of the most daunting factors concerning the world today. Most of the agricultural soil is slightly to moderately contaminated with heavy metals like Cd, Cu, Cr, Zn, Ni, Co, As and Pb. Both natural and anthropogenic activities like weathering of metalliferous rocks, volcanoes, erosion, combustion of fossil fuels, mining and industrial activities, use of phosphate fertilizers, sewage sludge, dust from smelters and bad watering practices in agricultural lands contribute to the heavy metal accumulation in the ecosystem (Yadav 2010; Su et al. 2014). The excess of heavy metals in soil affects the plant growth and root development. Roots significantly absorb higher amounts of heavy metals than the aboveground biomass and are thus more affected by heavy metals. There are mainly four mechanisms by which heavy metals exert toxicity in plants: (i) generating reactive oxygen species (ROS) in the plant cells which can damage various macromolecules (Møller et al. 2007; Pena et al. 2012); (ii) heavy metals having similarity with the nutrient cations compete with the nutrient molecules for absorption at root surface and interfere with cellular mechanisms, e.g. As and Cd show similarity with P and Zn, respectively, for absorption at the root surface (Monnet et al. 2001); (iii) heavy metals show high affinity for the sulfhydryl (-SH) group of various functional proteins, thus disrupting the structure and function and rendering them inactive; and (iv) heavy metals displace essential cations from their specific binding sites leading to cellular malfunctioning (Sharma and Dietz 2009; DalCorso et al. 2013; Singh et al. 2016). Inside the plant, these heavy metals interfere with various biological processes like reducing the photosynthesis, water and nutrient uptake and plant chlorosis growth inhibition, disturbing the metabolism of essential elements, disrupting the electron transport chain, disorder in cell membrane functions, suppressing root growth by decreasing the mitotic activities, etc. (Qadir et al. 2004; Yadav 2010). The level of toxicity depends upon various factors like concentration of heavy metals, duration of metal exposure and genotype of associated mycorrhiza and plant (Rajkumar et al. 2012).

Ectomycorrhizal fungi have been proven to protect the plants against the heavy metal stresses. The presence of ECM symbionts on plant roots significantly reduces the heavy metal uptake (Degola et al. 2015; Reddy et al. 2016). ECMs are not only capable of surviving under metalliferous soil but also promote the growth of host plant under metal stress. These fungi have developed specialized mechanisms to cope with heavy metals in the soil, thus protecting their host plant (Fig. 6.3). Primarily, ECM fungi restrict the entry of heavy metals through extracellular precipitation by excreting di-/tricarboxylic acids and oxalic acids, by biosorption of metal ions to the cell wall through chitin and glucosamine or by increasing the cellular efflux (Fig. 6.3). In spite of the restricted entry of these heavy metals, 20–30% of heavy metals could still be found in the cytosol and vacuoles (Blaudez et al.

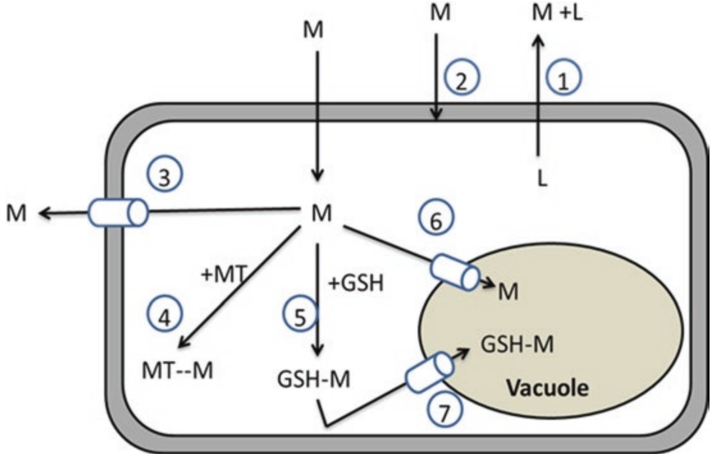

Fig. 6.3 Mechanisms of heavy metal tolerance in ectomycorrhizal fungi. (*1*) Extracellular chelation by secreted ligands, (*2*) cell wall binding, (*3*) enhanced efflux, (*4*) intracellular chelation by metallothioneins (MTs), (*5*) intracellular chelation by glutathione (GSH), (*6*) subcellular compartmentation, (*7*) compartmentation of GSH-M in vacuoles (Bellion et al. 2006)

2000). The heavy metals that enter inside the cell are chelated intracellularly through various thiol-rich compounds like metallothioneins (MTs), glutathione (GSH) and phytochelatins (PCs) (Fig. 6.3). Heavy metals have high affinity for the thiol group (-SH) of these chelators and form metal-MT, metal-(GSH) complex, which actively gets transported into the vacuoles (Sharma et al. 2016; Khullar and Reddy 2018). The role of ectomycorrhizal fungi in metal homeostasis has been clearly reviewed by Bellion et al. (2006), Luo et al. (2014) and Khullar and Reddy (2018).

ECM fungi modulate the heavy metal transfer into the host plants. The fungal mantle acts as an effective barrier or filter for the heavy metals to enter into the plant cells. The heavy metal concentration decreases from the rhizomorphs-hyphal mantle-cortical cells to vascular tissues. This has been clearly demonstrated in case of *Pinus sylvestris* colonized with *Suillus luteus* under zinc stress, where the concentration of zinc declined from 12,830 μg/g in rhizomorph to 2040–3820 μg/g in hyphal mantle, to 280–675 μg/g in cortical cells and to 430 μg/g in the vascular tissues (Turnau et al. 2001). Similar results were reported in case of Cu and Mn by Turnau et al. (2001), where the Cu concentration decreases from 420 μg/g in rhizomorphs to 17 μg/g in hyphal mantle and 6 μg/g in vascular tissues and Mn concentrations decreased from 490 μg/g in rhizomorphs to 88 μg/g in hyphal mantle and to 13–26 μg/g in cortical cells and 14 μg/g in vascular tissues (Turnau et al. 2001). *Betula pendula* seedlings inoculated with ECM fungi reduced the concentration of Cu and Pb in the above-ground parts of plant, thus protecting the plant from elevated metal stress (Bojarczuk and Kieliszewska-Rokicka 2010). Similar results have been reported in *Pinus sylvestris*, where the plants inoculated with *Suillus luteus*, *Suillus bovinus*, *Scleroderma citrinum*, *Amanita muscaria* and *Lactarius rufus* showed better plant protection and nutrient uptake, transferring less Cd, Cu,

Pb and Zn to the above-ground plants than non-mycorrhizal plants (Adriaensen et al. 2005, 2006; Krupa and Kozdrój 2007; Krznaric et al. 2009).

The reduced uptake of heavy metals by ECM fungi is due to the induction of efflux mechanism under heavy metal stress. Majorel et al. (2014) reported low accumulation of Ni in *P. albus* tissues, when exposed to high Ni concentrations, due to the metal efflux mechanisms. Similar observations were made in *S. bovinus*, *P. involutus* and *H. cylindrosporum*, where the increase in metal efflux resulted in low metal accumulation in the fungal tissue. This metal exclusion in fungal symbionts results in lower metal influx by the host plant, thus protecting from metal stress (Blaudez et al. 2000; Blaudez and Chalot 2011; Ruytinx et al. 2013). In contrast to the above observations, few studies have reported increase in metal bioaccumulation in both ECM and its associated host when exposed to high metal stress (Ma et al. 2014; Širić et al. 2016). ECM fungi *Paxillus involutus* significantly increased the net Cd^{2+} influx in the root apical region of *Populus canescens* when exposed to high cadmium stress (Ma et al. 2014).

ECM fungi have been reported to secrete various exudates in the rhizosphere in response to metal stress. These exudates like oxalic acid, formic acid, malic acid and succinic acid can alter the bioavailability of toxic metals in the rhizosphere, thus protecting the host plant from metal toxicity (Meharg 2003; Bellion et al. 2006; Ray and Adholeya 2009; Colpaert et al. 2011; Targhetta et al. 2013). Johansson et al. (2008) reported oxalate exudation in six ectomycorrhizal fungi *Hebeloma velutipes*, *Piloderma byssinum*, *Paxillus involutus*, *Rhizopogon roseolus*, *Suillus bovinus* and *Suillus variegatus*, when exposed to Pb, Cd and As stress (Johansson et al. 2008). In addition to fungal exudates, ECMs also induce plant exudates in the rhizosphere. *P. tabulaeformis* when colonized with ectomycorrhizal fungus *Xerocomus chrysenteron* showed enhanced activity of soluble proteins and acid phosphatases in root exudates than their non-mycorrhizal roots under Cu and Cd stress (Zheng et al. 2009). Bellion et al. (2006) reported up to 85% reduction in Cd uptake by oxalic acid exudation in cadmium-stressed *Paxillus involutus*. In a similar study, Shi et al. (2018) reported 99% removal of chromium Cr(VI) by *Pisolithus* sp1 by secreting H^+ ions and organic acids. The H^+ ions secreted by *Pisolithus* reduced the pH of medium, thus reducing Cr(VI). After 12 days of treatment with *Pisolithus*, it was observed that 75% Cr was removed due to extracellular reduction and 24% was removed by adsorption on cell wall (Shi et al. 2018).

Colpaert et al. (2011) reported development of adaptive heavy metal tolerance in different ECM fungi isolated from metal-polluted areas. *Suillus luteus* and *Suillus bovinus* isolated from metal-polluted areas showed better tolerance to heavy metals like Zn, Cu and Cd when colonized with *Pinus sylvestris* than the same fungus isolated from non-polluted areas (Adriaensen et al. 2005, 2006; Krznaric et al. 2009; Colpaert et al. 2011). Similar observations have also been made with *Pisolithus tinctorius*, *Pisolithus albus* and *Cenococcum geophilum* where the isolates from metal-polluted areas showed higher metal tolerance than the isolated from non-polluted areas (Egerton-Warburton and Griffin 1995; Gonçalves et al. 2009; Jourand et al. 2010).

ECM fungi have developed an intense intracellular mechanism to cope with the heavy metals accumulated inside the cell, so as to prevent their transfer to host plant. ECM fungi synthesize various thiol-rich ligands like metallothioneins and glutathione in response to metal stress (Khullar and Reddy 2018). Heavy metals having high affinity for the thiol group of these ligands bind them forming a strong metal-MT or metal-$(GSH)_2$ complex, rendering them nontoxic and subsequently sequestering into the vacuoles, thus protecting the host plant from metal stress. Differential expression of different metallothioneins and glutathione has been reported in response to different heavy metals. Reddy et al. (2014) characterized two MTs (*LbMT1* and *LbMT2*) out of six putative metallothionein genes from the genome of *Laccaria bicolor* and observed differential expression in response to copper, cadmium and zinc. Both metallothionein genes were induced under Cu stress, whereas only *LbMT1* was induced under Cd stress, and none of them was expressive under Zn stress (Reddy et al. 2014). Similarly in *Suillus himalayensis*, characterization of two metallothionein genes (*ShMT1* and *ShMT2*) revealed their potential role in Cu homeostasis, whereas no response was observed under cadmium stress (Kalsotra et al. 2018). This differential response of different metallothionein isoforms to different metals has also been reported in *Hebeloma cylindrosporum*, *Hebeloma mesophaeum*, *Amanita strobiliformis*, *Suillus luteus*, *Pisolithus albus*, *Russula atropurpurea* and *Paxillus involutus* (Bellion et al. 2007;Ramesh et al. 2009; Leonhardt et al. 2014; Sácký et al. 2014; Hložková et al. 2016; Reddy et al. 2016; Nguyen et al. 2017). However it was observed that most of the metallothioneins are responsive for Cu and Zn tolerance, showing very little response to Cd. However glutathione biosynthesis has been actively reported in response to Cd and As. Ilyas and Rehman (2015) reported that glutathione biosynthesis in ECM is mainly triggered by Cd and As followed by Cr, Pb and Cu. Courbot et al. (2004) reported increased production of glutathione in ECM *Paxillus involutus*, when exposed to cadmium stress. Similar observations were also made in *Laccaria laccata*, *Glomus mosseae*, *Funneliformis mosseae*, *Phanerochaete chrysosporium* and *Penicillium chrysosporium* (Gallie et al. 1993; Garg and Aggarwal 2011; Degola et al. 2015; Xu et al. 2015, 2016). Hence we can say that both metal chelators – metallothioneins and glutathione – play a key role in metal homeostasis in ECM fungi, thus protecting the host plant. However, a detailed study on what effect these chelators have on the metal toxicity in their host plant is still unexplored. These observations clearly demonstrate that the mechanisms involved in ECM protection of host plants are diverse. The heavy metal tolerance mechanism in ectomycorrhizal plants depends on various factors like type of plant, ectomycorrhizal strain and the type of heavy metal.

6.3.5 ECM in Nutrient Cycling in Host Tree

In the current scenario, bioavailability of inorganic nutrients like nitrogen, phosphorus and water in the forest soil is rapidly diminishing, resulting in retarded plant growth, poor photosynthesis and the loss of plant vitality. Most of the forest trees

rely on mycorrhizal symbiosis for fulfilling their nutritional requirements. The ECM fungi colonize the host plant, helping them to fetch nutrients from the deeper parts of the soil, which are otherwise inaccessible. There are mainly two pathways by which the mycorrhizal plants can uptake nutrients from the soil. First is 'the plant pathway' where the plant roots directly uptake nutrients from the soil through root epidermis and root hairs. This pathway is often limited by low mobility of nutrients in soils. The second pathway is 'the mycorrhizal pathway' where the nutrient uptake is mediated by the extraradical mycelium of fungus and transported to host plant through Hartig net (Bücking et al. 2012). However in ectomycorrhizal tree species, majority of the plant root is covered with thick mantle; therefore, the major nutrient uptake by plant is indirectly through the ECM. As reported in case of *Pinus*, 99% of the nutrition-absorbing root surface is covered with thick fungal mantle of ECM fungi (Taylor and Peterson 2000). However, the nutrient uptake mechanism in mycorrhizal plants depends upon the permeability of fungal mantle, its structure and other properties. If the fungal mantle is permeable, the roots can directly absorb the nutrients from soil, whereas if the fungal mantle is impermeable to nutrient ions, the underlying plant roots get completely isolated from the soil, extensively relying on the mycorrhizal pathway for nutrient uptake (Coelho et al. 2010).

During symbiosis, some ECM fungi secrete hydrophobins, which are small cysteine-rich amphiphilic proteins that foster the adhesion of hyphae to the surface and formation of aerial hyphae (Raudaskoski and Kothe 2015). ECM fungi *Pisolithus tinctorius*, *Laccaria bicolor*, *Tricholoma terreum*, *Tricholoma vaccinum* and *Paxillus involutus* have been reported synthesizing hydrophobins during ectomycorrhization (Tagu et al. 2001; Rajashekar et al. 2007; Plett et al. 2012; Sammer et al. 2016). Being hydrophobic, these protein molecules increase the water repellency of fungal mantle, thereby making it impermeable to both water and nutrients. The fungal mantle of *Hebeloma cylindrosporum* colonizing *Pinus banksiana* was found impermeable to sulphates, whereas that of *Pisolithus tinctorius* and *Suillus bovinus* colonizing *Pinus sylvestris* was poorly permeable showing retarded movement of nutrients across the membrane. However, the nutritional uptake by the host plants of all the three ECMs is completely under fungal control (Bücking et al. 2002; Taylor and Peterson 2005). The ECM pines are highly dependent on their fungal symbiont for both nutrition and water uptake (Ouahmane et al. 2009).

Phosphorus is the most important macronutrient required by plants for various cellular and metabolic activities; the availability of P in plants is highly dependent on the soil. ECM fungi facilitate the P uptake from the soil and transport it to the host plant. Due to the smaller hyphal diameter, ECM can scavenge orthophosphates (Pi) from the smallest core of soil and transport them to plant cortical cells. They also secrete various organic acids, phosphatases and phytases into the soil so as to facilitate P release from organic complexes (Alvarez et al. 2011; Plassard et al. 2011). The uptake of P from soil is facilitated by various phosphorus transporters. The expression of P transporters in ECM cell is regulated by various factors like availability of external P and demand in fungal cells (Bucher 2007; Plassard et al. 2011). Various phosphorus transporters have been identified in ectomycorrhizal fungi, which include five Pi transporters in *L. bicolor* (LbPht1;1 to LbPht1;5), two

in *H. cylindrosporum* (*HcPT1* and *HcPT2*) and seven in *Tuber melanosporum* (Tatry et al. 2009). ECM fungi have the tendency to store excessive P in the form of polyphosphates with average size from 10 to 20 Pi molecules, which allows the fungus to keep internal P concentration low, allowing efficient transfer from extraradical mycelium to the Hartig net (Viereck et al. 2004). Thus, the ECM fungi protect the host plant from P deficiencies.

The nitrogen transfer in mycorrhizal plants is also facilitated by their symbiotic fungi. ECM fungi can efficiently take organic and inorganic N from the soil and transfer it to the host plant. Many ECM fungi can mobilize and utilize amino acids and amides like glutamine, glutamate and alanine as N source for various metabolic pathways (Chalot et al. 1994). However, the inorganic nitrogen in soil is present in the form nitrate (NO^{3-}) and ammonium (NH^{4+}) ions. Among the two, ammonium ions are more efficiently taken up by the ECM than nitrate ions (Gachomo et al. 2009). It has been reported in ECM fungus *Hebeloma cylindrosporum* that the presence of NH^{4+} downregulates the expression of NO^{3-} transporters and nitrate reductase and up-regulates NH^{4+} transporters (*AMT1* and *AMT2*) (Javelle et al. 2003). This shows that uptake of NH^{4+} is preferred over NO^{3-}. Inside the fungal system, NO^{3-} is converted into NH^{4+} by nitrite reductase, which is further assimilated into amino acids by GS/GOGAT pathway (Tian et al. 2010). Earlier it was believed that the N can be transferred across the mycorrhizal interface in the form of amino acids or organic N. However Selle et al. (2005) identified high-affinity ammonium importer from *Populus trichocarpa* (*PttSMT1.2*), whose expression increased significantly when colonized with *Amanita muscaria*. This observation throws light on the fact that ammonium could be a major N source to be transported to plant by ectomycorrhizal fungi (Selle et al. 2005). The N transport mechanism from ECM fungi to the host plant was further explained in *Amanita muscaria*, where the exposure of NH^{4+} up-regulated the expression of high-affinity NH^{4+} importer (*AmAMT2*) in the extraradical hyphae but downregulated in Hartig net and fungal mantle (Willmann et al. 2007). The high expression of *AmAMT2* importer in extraradical hyphae indicates high NH^{4+} intake by the extraradical hyphae, whereas the lower expression at Hartig net would prevent the reabsorption of NH^{4+} by fungal hyphae from the root cells, thus increasing the net transport of NH^{4+} to the host (Willmann et al. 2007).

In exchange to all the nutrients, host plant rewards ECM fungi with its photosynthates. The ECM fungi form a strong carbon sink during the early symbiosis, thus receiving 20–30% of the total plant carbohydrate (Hobbie et al. 2008; Menkis et al. 2011). However the ECM fungi are not able to utilize the sucrose synthesized by the host plant due to lack of invertase gene which hydrolyses sucrose to glucose and fructose. In the *L. bicolor* genome, 15 genes encoding putative hexose transporters have been annotated; however, the genome lacks gene coding for invertase (Martin et al. 2008; Bonfante and Genre 2010). This clearly demonstrates that ECM fungi could not hydrolyse sucrose; therefore it depends upon the host plant to release glucose and fructose. It has been reported that *L. bicolor* could uptake only glucose from the host plant, whereas *Amanita muscaria* and *Hebeloma cylindrosporum* could even metabolize fructose (Bonfante and Genre 2010). Nehls et al. (2001)

reported that glucose is mainly taken up at the Hartig net, whereas fructose is taken up by hyphae of inner mantle layer. During symbiosis, the expression of hexose transporters increases immediately, indicating that the fungus is highly dependent on glucose from mycorrhizal interface (Lopez et al. 2008; Bonfante and Genre 2010). It has been observed that the P and N uptake in ECM fungi is stimulated by the supply of carbon from plant host. The Pi and N efflux from the fungus could directly be linked with the glucose uptake by the mycorrhizal fungi (Bücking and Heyser 2003; Bücking 2004; Kytöviita 2005).

6.4 Conclusions

Ectomycorrhizal fungi are a heterogeneous group of diverse fungal taxa, associated with more than 50,000 plant species. These symbiotic fungi play an important role in tree sustainability and ecosystem development. They protect the host trees from various biotic and abiotic stresses like heavy metals, salinity, drought, nutritional imbalance, pathogens and pests. Different ectomycorrhizal fungi respond differently to different stresses, thus balancing the whole ecosystem. The high taxonomic and functional diversity of these ECM fungi delivers a reliable support to trees in tolerating large spectrum of ecological niches.

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7 Diversity of Arbuscular Mycorrhizal Fungi in Relation to Sustainable Plant Production Systems

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Abstract

Arbuscular mycorrhizal fungi (AMF) have been suggested as an important component of sustainable plant production systems, where restoration and conservation of resources, including environment, is the mainstay. Present chapter highlights recent developments on AMF diversity with reference to its importance for sustainable production systems. The important aspects of taxonomic, genetic and functional diversity of AMF in relation to host-plant interaction, plant nutrient uptake, growth and productivity benefits along with soil nurturing properties such as aggregation are discussed. Influence of several agronomic practices on diversity of these fungi is reviewed with reference to its potential for future exploitation as a commercial biofertilizer.

Keywords

Arbuscular mycorrhizal fungi · AMF diversity · Plant responses · Soil management practices

7.1 Introduction

A sustainable plant production system encompasses restoration and conservation of resources including soil, water and air quality (Rillig et al. 2016). The main goal of a sustainable production system is to create convergence of society's all requirements without jeopardizing the ever-limited resources available for future

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generation and integrate the ecological wealth with social and economic balance and profitability (Siddiqui and Pichtel 2008; Rillig et al. 2016). Several soil factors contribute to the sustainability of agricultural ecosystems via controlling soilborne diseases, increasing soil microbial activity and balancing the nutrient cycles (Bender et al. 2016). Mycorrhizal associations between roots of higher plants and fungi have been well documented for its profound effect on sustainable plant production systems (Rillig et al. 2016). Building sustainable agri-production systems using mycorrhizas lays a focus on utilizing natural resources to gain higher levels of food productivity with minimum uses of chemical fertilizers, pesticides and environmental pollutants without compromising the soil health and quality.

Various types of mycorrhizas are known to occur in most land plants, which include 72% arbuscular mycorrhiza (AM), in comparison with 2, 1.5 and 10% for ectomycorrhizal, ericoid and orchid mycorrhizas, respectively (Van der Heijden et al. 2015; Brundrett 2017). Different types of mycorrhiza produce different structures in the roots and involve different hosts of the plant family, for example, ectomycorrhiza develops an association with the lateral root of most gymnosperms and produces penetrative labyrinthine hyphae and hyphal mantle (Brundrett 2017). Orchid mycorrhizas, on the other hand, forms symbioses between roots of family Orchidaceae of higher plants and fungi belonging to class Basidiomycetes. In ericoid mycorrhizas form symbioses with the roots of host plants of Ericaceae and hyphae of most ascomycetous fungi which benefit the nutritional quality of soil and host plants.

Much has been published about the role of AM in sustainable production systems, and the fungi forming them are called as arbuscular mycorrhizal fungi or AMF. These fungi were earlier placed in the phylum *Glomeromycota* (Schußler et al. 2001) but later were shifted to subphylum *Glomeromycotina* (Spatafora et al. 2016). These fungi have played a crucial role in the evolution of land plants for over 460 million years (Remy et al. 1994) and currently inhabiting the roots of most land plant species and bryophytes (Smith and Read 2008). The association of AMF positively influences host plant in growth, nutrient absorption, disease and pathogen resistance and resistance against other abiotic factors such as salinity, pollution, drought, chilling, etc. (Smith and Read 2008; Gupta et al. 2018a). AMF are one of the most abundant organisms that can be found within the broadest range of habitats with important roles in plant productivity.

In recent studies and forums, AM symbiosis and diversity had been discussed in the context of sustainable production systems because of various reasons including (i) its dominance over most crops and approximately 2,00,000 host plant species across all habitats (excluding Brassicaceae members) (Lee et al. 2013); (ii) its positively multifunctional roles in soil structure preservation, plant nutrition and abiotic and biotic stress tolerance (Smith and Read 2008; Leifheit et al. 2014); (iii) negative effects of tillage, use of fertilizers and non-host crops on AMF diversity and abundance, thereby affecting its potential functioning; and (iv) management of AMF in increasing the plant productivity.

In this chapter, AMF diversity is discussed with reference to sustainable plant production systems. Besides revisiting the contribution of AMF in mineral nutrition

and increasing the overall growth of crop plants, the agronomic practices, e.g. crop and soil management practices, fertilizers, etc., as key factors influencing the AM population and functioning are also discussed in this chapter. Moreover, recent aspirations of application of the current knowledge about these fungi to gain sustainability in agriculture to augment plant production and the associated ecological implications are also discussed. In this context, the supporting examples from natural environments are also cited along with agroecosystems.

7.2 Fungal-Root Interaction for Mycorrhiza Formation

AMF and plant communication starts with the secretion of signalling molecules (strigolactones) by the host in the root exudates (Bouwmeester et al. 2007; López-Ráez et al. 2011). The strigolactones received by AMF increase metabolism resulting in the development of pre-symbiotic phase in fungus characterized by profuse hyphal branching, which improves root contact and enhances symbiosis (Akiyama et al. 2005). The AMF inoculum entities which include soil-based propagules, viz. spores, extraradical hyphae and mycorrhizal root fragments, allow AMF to colonize the new plant roots by establishing the developed mycelial network in soil (Muller et al. 2017). AMF produces diffusible fungal signals known as 'Myc' factors inducing symbiosis (Parniske 2008; Bonfante and Genre 2010). In the presence of root, hyphae proliferate in the soil to establish contact and form appressoria, by producing swelling in the epidermal cells. Hypha facilitates itself through appressoria penetrating epidermal cells to enter the root which crosses through epidermal cells to start branching in the outer cortex (Brundrett et al. 1985). Aseptate hyphae spread in the cortex in every direction available from the entry point, cross walls and proliferate linearly or in coils (Smith and Smith 1997).

Characteristic branching structures within cells, called arbuscules, are produced in the cortex region, act as a site of nutrient exchange (Smith and Read 2008). They are responsible for transferring carbohydrates from the plant tissues to the fungus and supplying nutrients and water from the AMF to the plant (Rich et al. 2017). Vesicles are formed inside the root, inter- and intracellularly which act as storage structures. Vesicles become thick-walled and act as propagules in older roots (Brundrett 2008). AMF also produce a mycelial network outside the root called as the extraradical mycelium, where spores and sporocarps are produced along with mycelium that serves as propagules to complete the life cycle (Barea et al. 2013, Gupta 2017).

7.3 AMF Diversity and Why It Is Important in Sustainable Production Systems?

The term 'biodiversity of AMF' comprehended the totality and variability of species (taxonomic), genes (genetic) and the ecosystems they occupy (functional). Traditionally, the biodiversity determination of AMF has been based on spore

morphology (Morton 1988; Morton and Benny 1990; Blaszkowski 2012). As far as the taxonomy of AMF is concerned, the accurate identification of AMF has been challenging. The classical or morphological determination of spore diversity is often not accurate because very few morphologically distinguishable characters are present in the spores of AMF. In addition, one species sometimes produces more than one morphological feature (Lee et al. 2013). The genetic studies based on rRNA sequences contradict the morphological classification, and we require a robust classification system. The presence of genetically different isolates even within one AMF species has been demonstrated experimentally by Lee et al. (2013). Thus, the determination of taxonomic biodiversity is jeopardized.

According to the latest estimates, we presently have +/−300 species of AMF reported from all over the world, 50 of which are discovered in the last 10 years (Opik et al. 2013). In India, more than 161 species have been known to exist up to 2016 (Gupta et al. 2017). The studies from India showed that *Funneliformis mosseae* is the most widely distributed species and *Glomus* is represented by the highest number of species (Gupta et al. 2014). The other widely distributed species in India are *Funneliformis mosseae*, *Rhizophagus fasciculatus*, *R. intraradices*, *Acaulospora laevis*, *A. spinosa*, *A. scrobiculata*, *Glomus aggregatum*, *Claroideoglomus claroideum* and *C. etunicatum*. However, most of this information of diversity is based on morphological characteristics of AMF rather than molecular methods (Gupta et al. 2014). The fact was revealed when complete database of AMF diversity and distribution was built with specific information on their mode of diversity determination.

AMF diversity in any ecosystem is often not accurately determined. Re-evaluation of species number in any ecosystem based on molecular methods has indicated that the actual AMF species number in the ecosystems is much higher than what is revealed in literature (Opik et al. 2013). To date, roughly 300 species of *Glomeromycota* have been described based both on morphology and molecular data (rRNA sequences) with molecular studies indicating that there are between 300 and 1600 taxa (Van der Heijden et al. 2015; Taylor et al. 2017). There are several problems with determination of molecular diversity as well (Taylor et al. 2017) such as AMF have been known to reproduce only by asexual spores and their coenocytic hyphae are distributed throughout the soil. We have very little idea about the size of the individuals of these fungi or how much genetic diversity occurs within their multinucleate organs. The coenocytic hyphae enclose hundreds of genetically different nuclei and maintain a stable assemblage of several different genomes during the life cycle (Tisserant et al. 2012), but this genomic organization has been questioned and needs to be studied further.

The other problems associated with AMF diversity and abundance determination are due to their hidden lifestyle inside the roots and soil. They produce hyphae, spores and auxiliary bodies in the soil and hyphae, arbuscules and vesicles in the roots. They are obligate symbionts and cannot be cultured in artificial media. The only practical viable method to conserve them is to maintain in pot cultures. Maintaining them in pot culture is a lengthy and cumbersome process. It at least requires 4–5 months to raise the culture of one isolate. Further to examine genetic

diversity requires establishing single-spore cultures that require several steps of purification. Currently there are about 228 to 230 valid species (of the ~ 300 formally described) of which only 81 (35%) are propagated in culture collection centres at INVAM (International Culture Collection of Vesicular Arbuscular Mycorrhizal Fungi), BEG (La Banque Européenne des Glomales) and GINCO (Glomales In Vitro Collection).

Species richness, diversity and distribution of AMF or Glomeromycota over different habitats have been extensively studied in recent times (Davison et al. 2015; Casazza et al. 2017). The presence of AM in almost all terrestrial habitats (Smith and Read 1997) along with low specificity in root-fungus interaction (Feddermann et al. 2010) makes functional diversity determination of these fungi more important. Functional diversity of AMF is much less studied compared to structural or genetic diversity. Only few AMF species have been investigated for functional diversity across drastically different environments which include *Glomus intraradices*, *Glomus mosseae* and *Gigaspora margarita* (Smith and Read 1997). Therefore, functional studies of AMF often are biased towards fungal species that are well known to benefit their host plants or are very abundant or are easily detected in culture systems (Opik et al. 2009). Few functional studies on diversity recently have demonstrated that there is high variability in the establishment of symbiosis in different combinations of host plant and AMF (Munkvold et al. 2004). Understanding of functional diversity of AMF is important since different combinations of host plant and AMF will have different ecological implications in terms of nutrient availability and ecosystem functioning.

Assessment of AMF diversity and abundance of AMF in the agricultural field is an important component of mycorrhizal technology, which deals with the application of mycorrhizal fungal inoculum for increasing crop productivity (Solaiman et al. 2014). In any agroecosystem AM is not a homogeneous association. Rather it's a plant-fungus combination, characteristics of which depend on the partners involved (Lee et al. 2013). Also, each AM formation is a phenotypic response of plant and fungal genes and their interaction with the environment. Each AMF-plant association is unique not only in morphology but also in the extraradical mycelium, nutrient uptake and transfer capacity (Lee et al. 2013). Thus more detailed and extensive studies are needed for predicting the functional performance of species or genera (Lee et al. 2013; Cavagnaro et al. 2015).

The role of AMF in increasing nutrient uptake, tolerance to heavy metals and saline conditions and protection from soilborne diseases is of little ecological significance if diversity of local AMF was not considered (Hart et al. 2017). The correct understanding of the local AMF biodiversity could be used to reduce the input costs in agricultural production systems and taking them towards sustainability. The economic aspect of AMF diversity and sustainability is discussed separately in Sect. 6 on the future application of mycorrhizal research in sustainable agriculture.

7.4 Are AMF Wonder Fungi for Crops?

The AMF associations are known to occur in the roots of most agricultural plants. AMF forms symbiosis with some most important crop plants such as *Triticum* (wheat), *Glycine max* (soybean), *Phaseolus vulgaris* (beans), *Solanum lycopersicum* (tomato), *Sorghum bicolor* (sorghum), *Capsicum annum* (red peppers) and *Daucus carota* (carrot) (Opik et al. 2010). Few strains of AMF such as *Glomus intraradices* have been domesticated more popularly and are available with numerous commercial mycorrhiza-based inoculants, and the application of these by farmers is increasing (Gianinazzi 2014; Hijri 2016). The role of AMF in maintaining and improving plant productivity is performed in various ways, including improving the nutrient availability, water absorption, protection against pathogens, tolerance

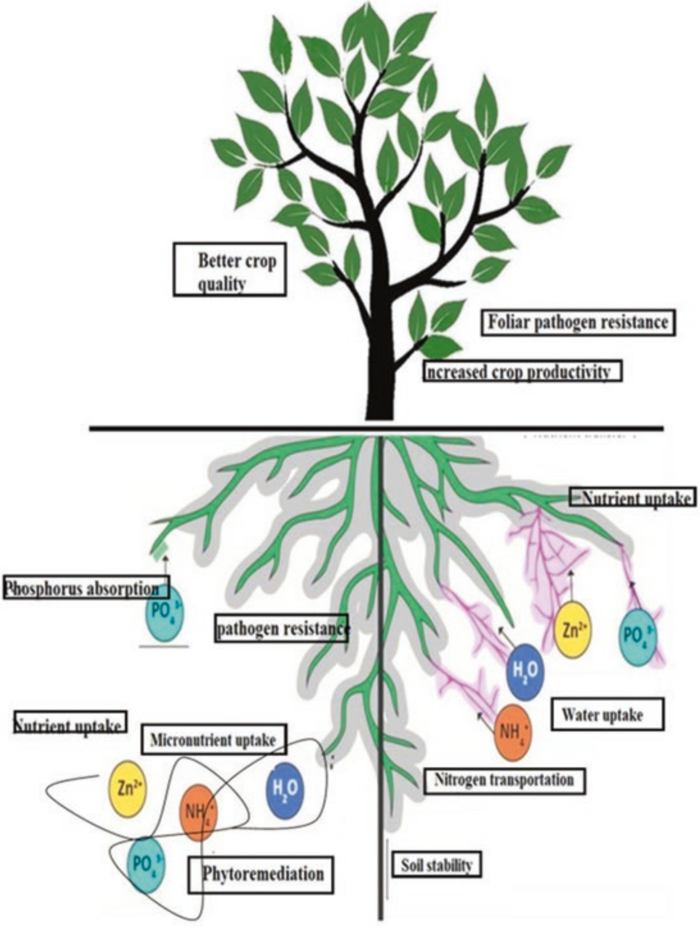

Fig. 7.1 Mechanisms of AMF contribution to sustainable host plant development

against heavy metal stresses, salinity and drought, soil structure and aggregation (Giri and Mukerji 2004; Harrier and Watson 2004; Nichols 2008; Smith and Read 2008; Smith et al. 2010; Cavagnaro et al. 2015; Yang et al. 2016). The role being played by these fungi depends on the situation and plant-fungus combination.

AMF symbiosis contributes to sustainable plant production systems by three main ways (Verbruggen et al. 2010): firstly by serving as a desirable nutrient sink in which mycorrhizal plants have a higher growth and yield, secondly by mycorrhizal crops having better nutrient extraction and uptake capacity, and thirdly by altering the soil properties such as soil aggregation. Thus, AMF imparts growth and productivity benefits to crops by increasing favourable microbial interactions and resistance to unfavourable abiotic conditions (Lenoir et al. 2016; Liu et al. 2007). Several abiotic stresses including nutrients P and N, micronutrients and moisture stress and biotic stresses like diseases by plant pathogens and competition of survival within the plant species are summarized in Fig. 7.1. Specific examples of growth benefits of AMF species inoculation in crop plants, along with their mechanism of action are given in Table 7.1 and each benefit is discussed in details in the next section.

7.4.1 AMF-Host Nutrient Exchange

AMF contribute to the plant growth by improving its nutrition of macro- and micronutrients. Some selected examples are cited here:

(a) **Phosphorus absorption**: Many studies have suggested that inoculation with AMF could achieve a reduction of up to 80% of the recommended phosphate fertilizer dosage (Hijri 2016). Symbiosis between AMF and host plant provides P to the plant from rhizosphere via arbuscules (Takanishi et al. 2009). The extraradical hyphae uptake phosphate ions against a concentration gradient (Tani et al. 2009). The polyphosphate molecules are then transported with the help of

Table 7.1 Few examples of growth benefits of inoculation with AMF species in various crop plants

Host plant	AMF species	Reasons	References
Trifoliate orange	*Funneliformis mosseae*	Induce root hairs by increasing IAA level	Chun et al. (2018)
Cowpea	*Glomus deserticola*; *Gigaspora gigantea*	Protection against charcoal rot	Oyewole et al. (2017)
Robinia pseudoacacia	AMF	Phytoremediation	Yang et al. (2016)
Tomato	*Glomus mosseae*; *G. intraradices*	Protection against *Phytophthora parasitica*	Cordier et al. (1998)
Trifolium subterraneum	*Glomus mosseae*	Cadmium tolerance	Joner and Leyval (1997)
Canola and clover	*Glomus intraradices*; *G. versiforme*	Shoot dry weight	Marschner and Timonen (2005)

high-affinity Pi transporter proteins to the symbiotic interfaces located in root cortical cells, where Pi is released into the plants (Basu et al. 2018). The AMF symbiosis allows better plant phosphorus uptake from soils with low levels of available P (Van der Heijden et al. 2015). The volume of soil explored by mycorrhizal roots is increased manyfold because of the network of fungal mycelium connecting to AM roots making a mycorrhizal root more efficient in phosphate uptake.

(b) **Nitrogen transportation**: The AMF hyphae stimulate other rhizosphere organisms to mineralize organic N and make it available to host plants (Hodge et al. 2001). Mycorrhiza helps in the uptake of N from soil and increases the utilization of different forms of N (Hawkins 2001; Jacott et al. 2017). AMF can directly take up the N or supply of nitrate and ammonium by active transport also (Basu et al. 2018). Thus in sustainable agricultural systems, an effective AMF association could increase the benefits to the leguminous crops in rotations and the utilization of compost and other organic nutrient sources (Bucking and Kafle 2015).

(c) **Micronutrient absorption**: Besides improving the availability of major nutrients, crop quality is enhanced due to improved acquisition of micronutrients by AMF (Hart and Forsythe 2012; Pellegrino and Bedini 2014). A study carried out based on meta-analysis of data available in literature concluded the AM mediated positive assimilation of zinc concentration in various crop plants under certain conditions (Lehmann et al. 2014; Hart et al. 2017). AMF enhance acquisition of copper, manganese, iron, sulphur, potassium, calcium, magnesium and aluminium in host plants of mycorrhizal symbiosis (Hart et al. 2017); however, the degree of micronutrient absorption from soil depends on pH and other properties of soil. In acidic pH conditions of soil, AMF have properties of ameliorating aluminium and manganese toxicities for host plants (Clark and Zeto 2000).

7.4.2 AMF Role in Soil Nurturing

(a) **Soil aggregation**: Fungal symbionts develop a network of extraradical hyphae into the soil making up to 50% of fungal mycelium in soil, hence representing a major portion of microbial biomass in the soil (Leake et al. 2004). AMF has an important role in soil aggregation by secretion of glomalin, a glycoprotein secreted from growing hyphae, which leads to soil stability (Riling et al. 2002, Bedini et al. 2009; Riling and Mummey 2006, Lee et al. 2013). It is suggested that glomalin helps in soil aggregation by increasing physical enmeshment of soil particles and cementing them with sticky exudates. This results in reduced erosion and leaching losses in the soil, thus efficient nutrient storage (Tisdall 1991; Wright and Upadhyaya 1996; (Rillig and Mummey 2006; Querejeta et al. 2009).

(b) **Heavy metal toxicity**: Plants employ various mechanisms to maintain metal ion equilibrium. AMF trace toxic elements by sequestration and help host plant to survive by reduction in their tolerable value (Manjhi et al. 2016; Dong et al. 2008). AMF has also been observed by many scientists to provide protection against heavy metal toxicity in fields against such as copper, cadmium or arsenic (Silva et al. 2006; Dong et al. 2008; Schutzendubel and Polle 2002). This is suggested to be achieved either by elevating nutrient uptake like N and P (Senés-Guerrero and Schüßler 2016) or by other mechanisms which include diluting or cleaning of heavy metals from soil.

7.4.3 Other Indirect Mechanisms

(a) **Root exudates**: Initiation of symbiosis triggers plants to secrete more exudate organic acids, polysaccharides, phenolic compounds, amino acids and enzymes like phosphatases (Gupta et al. 2018a, b) from the root which are beneficial for the growth of AMF and host plant themselves. This also has an influence on the associated microbiome functioning.
(b) **Secondary metabolites**: AM symbiosis has been suggested to stimulate the synthesis of several secondary plant metabolites including polyphenols, phytosterols, vitamins, lignins, terpenoids and carotenoids for increased plant tolerance to abiotic and biotic stresses (Hooper and Cassidy 2006; Stan et al. 2008; Seeram 2008). After associating with AMF, defence responses of plants accelerate the formation of phytotoxins, chitinases, glucanases, peroxidases, PR proteins, phenolics and hydroxyproline-rich glycoproteins (Smith et al. 2010).
(c) **Pathogen resistance**: AM associations reduce the loss caused by various soil-borne pathogens including *Fusarium* and *Phytophthora* (Jacott et al. 2017). Several studies suggest that the pathogen resistance property of AMF can be utilized as biocontrol feature like other fungal biological control agents (Aguilar and Barea 1997).

7.4.4 The Contradictions: Growth Reduction Reports

Mycorrhizal-mediated growth reduction has been reported in many crops by several researchers (Graham and Abbott 2000; Dai et al. 2014; Ryan et al. 2005). This reduction in growth has been attributed mainly to reduction in the stored carbon of the host plants. It is suggested that since the plants are unable to adjust their carbon assimilation rate to accommodate the requirements of AMF, this results in a reduction in growth (Koide and Elliott 1989). Nutrient resources are diverted to associated rhizobia and mycorrhizal fungi which increase their individual fitness resulting in growth depression in the host plant (Kier 2015). However, unlike rhizobia there is yet little evidence that inoculation by commercial AMF is useful in agricultural

systems and a growth benefit of crops of the extent as achieved by conventional fertilizers can be achieved by such inoculations (Hart et al. 2017). There is a need for further systematic study to address these questions.

7.5 Maintaining AMF Diversity and Functioning for Sustainable Production

For the production of a sustainable agroecosystem and to receive benefits from AM associations, emphasis must be on green practices that support the occurrence and functioning of soil organisms. It has been shown that several agricultural practices and anthropogenic activities directly or indirectly influence the diversity and abundance of AMF in soil (Gupta et al. 2018b). Conventional farming relying on tillage and application of chemical fertilizers and fungicides result in large ecological disturbances and thus affect the functioning of AMF and may not be sustainable in the long term. This has been documented in many studies that these activities affect the diversity and abundance of AMF communities represented both as spore and mycelium in temperate and tropical environments (Sieverding 1990; Gupta et al. 2018). Several examples are cited below, which substantiate that before AMF are applied as bioinoculants, the agricultural practices need to be tuned to be effective in sustainable plant production.

7.5.1 Tillage Practices

Tillage practices of every type including digging, stirring or overturning done for soil preparation negatively affect the mycorrhizal hyphal network. It has been observed that tillage reduced AM colonization in *Phaseolus vulgaris* L. and other crop plants (Mulligan et al. (1985). Mycorrhizal root colonization of corn growing in no-tilled and ridge-tilled plots was greater than that in conventional-tilled plots (McGonigle and Miller 1996). Since mycorrhizal hyphal network and other fungal structures are not disturbed, no-tillage often stimulates mycorrhizal functioning and nutrient uptake in soil (Dodd 2000).

7.5.2 Manures and Fertilizers

Application of organic fertilizers and farmyard manure is shown to increase densities of AMF spores (Harinikumar and Bagyaraj 1989). Chemical fertilizers in contrast decrease AMF diversity and functioning. Different AMF species are affected differently. Spores of *Glomus* spp. were not much affected and were abundant whether fertilized with mineral or organic fertilizers (Oehl et al. (2004), but spores of *Acaulospora* and *Scutellospora* species were more in number in soil that only received organic fertilizers. Gupta et al. (2018) found that spores of *Glomus constrictum* survived the hazardous environmental conditions, but the large-sized spores were much less frequent.

7.5.3 Fungicides

Use of fungicides especially systemic ones has been shown to affect the occurrence and functioning of the AMF (Menge et al. 1978). It affects several phases of AMF life cycle such as reduced sporulation and percent root length colonized. The effect of fungicide on AMF, however, varied with environmental conditions AMF-fungicide combination (Turk et al. 2006). Mode of fungicide application also made a difference like soil applications of fungicides were observed to promote AM colonization in corn and soybean (Groth and Martinson 1983), but fungicide captan when applied on seeds had no effect (Kucey and Bonetti 1988). It reduced symptoms and occurrence of pathogen *Fusarium solani* when captan was applied with AM inoculum in case of *Phaseolus vulgaris* plants (Muchovej et al. 1991). Application of fungicides as soil drenches also reduced AMF colonization. This was true with many fungicides, such as benomyl, captan, pentachloronitrobenzene and emisan (Schreiner and Bethlenfalvay, 1997). In non-fumigated soil, seed-applied fungicides (fludioxonil) appeared to promote AM colonization (Murillo-Williams and Pedersen 2008). This fungicide was applied to target aggressive pathogens like *Rhizoctonia* spp.

7.5.4 Crop Rotation, Cover Crop and Farming Systems

Besides chemicals, AMF functioning and species compositions also get affected by the type of agricultural system. Mader et al. (2002) compared long-term effects of conventional and organic farming for more than 22 years. It was reported by them in an earlier study in Switzerland that organic system of farming favours AMF colonization up to 40% and more (Mader et al. 2000). Soils used for agricultural production are often dominated by *Glomus* species (Oehl et al. 2003) and have a low diversity of AMF as compared with natural ecosystems (Menendez et al. 2001). Crop rotation significantly changes the species composition in AMF spore communities in case of soybean (Mathimaran et al. 2007). There is up to 13% reduction in mycorrhizal colonization in roots after 1-year rotation with a non-mycorrhizal crop (Harinikumar and Bagyaraj 1989). Fallowing, however, reduced AMF colonization by 40%. Similarly, the crop used for rotation also made a difference like in a study by Bagayoko et al. (2000), higher AM colonization in cereals sorghum and pearl millet was reported when in rotation with legumes (cowpea, groundnut) than in continuous cropping.

7.6 Future Application of Mycorrhizal Research in Sustainable Plant Production

The sustainable agriculture depends on continuous rotational crops, use of biofertilizers and nutrient cycling (Mader et al. 2002; Bender et al. 2016). To make use of mycorrhizal technologies for sustainable ecosystems, we need to clearly understand

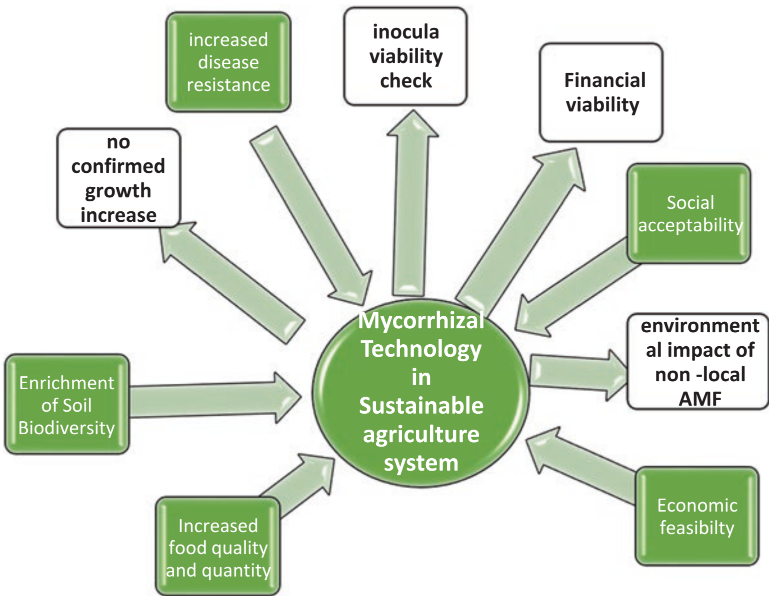

Fig. 7.2 Evaluation of AMF application in sustainable agricultural system with reference to its diversity; the arrow towards the centre depicts the positive aspects and the arrow away and the white box indicate limitations

the contribution of mycorrhiza in nutrient cycles and critically evaluate its positive and negative aspects. Several parameters, which must be considered before recommending AMF inoculant to farmers, are summarized in Fig. 7.2. Much remains to be learned about the biology and ecology of these fungi before we can adequately apply AMF biofertilizers for sustainable production.

7.6.1 Environmental Impact of Adding a Commercial AMF Inoculant

Environmental impact of adding any AMF inoculum into a region from which it was not isolated needs to be thoroughly considered before applying commercial inocula (Schwartz et al. 2006; Hart et al. 2017). Hart et al. (2017) had suggested two important tests that need to be performed before any AMF inocula are applied at commercial level: first, an assessment of its impact on structure and diversity of the native AMF community and, second, a population genetics-based assessment of introduction of a number of new alleles into the local AMF inoculant population (Ceballos et al. 2013). Recently, Hart et al. (2017) concluded that the current practice of AMF inoculation for growth and productivity increase is not appropriate ecologically as most systems already contain functional AMF communities. We need to check the competitiveness of inoculant AMF with that of local fungi before

suggesting its exploitation. For example, a degraded system may already contain a highly competitive AMF that has been proven to be tolerant to local conditions. In addition, it has yet not been demonstrated that certain strains of AMF are superior fungal mutualists in comparison to others. Hart et al. (2017) suggested that natural inoculum with native fungal diversity from local soils should be preferred to commercial inoculants. AM inoculation research should be carried out in closed systems using artificial soil or hydroponics. Dependency and specificity in the association between partners can also influence the distribution of real plants.

7.6.2 Bottlenecks in a Realistic Agricultural Management System

There are several limitations in application of AMF inoculants in sustainable production systems, some of which are highlighted below:

(b) **No confirmed growth increase**: AMF inoculation has been demonstrated to have increased plant growth in many plant species and is duly cited in several scientific publications (Smith and Read 2008). However, there is a need to perform these demonstrations in an agriculturally realistic situation such as unsterilized soil or the soil that already contains native AMF strains. Also, in many studies AMF inoculation has not produced comparable yield increases in a realistic agricultural management system. For example, inoculation in rice with *Rhizophagus irregularis* resulted in up to fivefold increase in rice growth in the greenhouse (Angelard et al. 2010), but no effect could be demonstrated in field conditions. In this respect, cassava is one of the only crops that have been shown to benefit from AMF inoculation in field conditions (Ceballos et al. 2013). On the contrary, there have been several growth reduction reports as discussed in the earlier section.

(c) **Financial viability**: AMF have not yet been applied on a wide scale because of financial constraints. To achieve the crop yield of the same level as obtained by commercial fertilizers, it required inoculating plants with a large amount of AMF inoculum. This inoculum producing unit needs to be located at the farm itself; otherwise, supplying the required amount of inoculum for a farming unit would turn into very expensive practice taking into consideration transportation and storage costs. Thus, financial viability of using microbial inoculant production is also posing a constraint in making its application sustainable.

The economic analyses are a must in sustainability studies as it would allow us to formulate achievable targets. In vitro-produced *Rhizophagus irregularis* is the most commonly applied AM fungus for this purpose. Cost considerations require researches on determination the minimum inoculum quantity needed to be applied. For example, in a study on cassava, each plant was to be given up to 12,500 propagules of the inoculum (Ceballos et al. 2013) to achieve desired growth increase. In contrast, in the greenhouse we need to inoculate plants with only 300 spores of *R.*

irregularis to obtain desired colonization and growth response. In addition, it is likely that the same inoculum may be effective in more than one season. Recently Rosikiewicz et al. (2017) devised two modified culture systems that allowed high spore production with low rates of contamination. We need many more such studies with a focus on economic analysis.

c. Other considerations: There needs to have a standardized method for testing the inoculum quality and specifications. A lot remains to be explored about several aspects of these fungi and needs to induct more types of AM species other than commercially exploited AMF species, e.g. *Rhizophagus irregularis*, for large-scale uses and production of AMF biofertilizers for application in sustainable plant production.

Another aspect to consider is the parameters that influence the effectiveness of mycorrhizal colonization and abundance. Specially, parameters influencing mycorrhizal symbiosis negatively need to be studied as well together with the positive factors. For example, the strategy for evaluating efficient AMF based on enhanced P availability in target plants needs to be promoted than the ones based on growth responses and colonization parameters (Sharma and Adholeya 2015). The use of AM signature fatty acids 16:1ω5c neutral and phospholipids as AM-specific biomarker can be employed while assessing the efficacy of AMF for the purpose of AM production and to assess the sustainability of long-term cropping systems when compared to use of conventional microscopic methods (Sharma and Buyer 2015).

Study of functional diversity and characteristics of AMF species related to host-plant selection, response to different environments and growth response of specific host plants could be better understood when the genetic diversity of AMF is clearly established. Rillig et al. (2016) summarized three directions of further research that are crucial for applications of AMF inoculation for sustainable agriculture. First, better understanding of the role of mycorrhiza in every aspect of sustainability is required, including clearer view of the ways by which mycorrhiza makes an agroecosystem sustainable and interacts with soil biota. This can be achieved by measuring all other organisms which are important in soil aggregation (bacteria, saprobic fungi and soil animals) and nutrient cycling (nitrifiers, phosphate mobilizers, ammonia oxidizers and non-symbiotic and symbiotic azotrophs) as well as assessing other factors that influence their overall contributions in crop quality and yield increase. Defining influential parameters of mycorrhizal effectiveness is the second priority. This would help the agricultural management practices to not obstruct mycorrhizal benefits to crop plants. Third direction needs the expansion of variables for documentation of effects of mycorrhiza on host plant. For instance, data for plant growth promotion exists but lesser knowledge of nutrients and micronutrients involved in this (Lehmann et al. 2014; Lehmann and Rillig 2015).

7.7 Conclusions

Modern era has increasing demands of organic farming, quantitative and qualitative crop plant production and globally approved agro-systems. Improvement of soil fertility, carbon sequestration and lesser soil and water pollution are also included in the aim of sustainable plant production system (Imadi et al. 2016). Conservation cultural practices like crop rotation, minimum tillage and inter- and mixed cropping help a great way in sustainable plant production. The number of microorganisms including bacteria, fungi, actinobacteria and protozoa plays a role in increasing plant growth and improving soil fertility (Hart et al. 2017). AMF being obligate symbiont, its functioning and population is greatly affected by soil disturbance and cropping sequences. Inclusion of potential hosts for maintaining high populations of indigenous AMF such as maize in the rotation cycle would help in functioning of AMF as biofertilizer (Sharma et al. 2012). Moreover, reduced tillage, manures and cover crops are recognized as a practical way to maintain high population of functional effective AMF eventually to support sustainable crop production. The AM hyphal networks distribution in soil helps in the improvement of soil fertility by secretion of glomalin that helps in soil aggregation by enhancing water holding capacity and water retention. AMF also confers resistance to plants against biotic and abiotic stresses. AMF-assisted bioremediation also enhances phytoremediation through promotion of plant growth. It also enhances organic acid synthesis and chelation of heavy metals by immobilizing metal ions by AMF roots or hyphae. Hence, there is need to modulate the mycorrhizosphere to manage resident AMF for improving the plant health. Sustainable cropping sequences coupled with minimum tillage practices should be the key aim of the applied AMF research to sustain higher productivity of plants without compromising the soil health.

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8 Microbial Diversity on Grapes and Other Fruits: Role and Significance in Fermentation

M. V. Deshpande

Abstract

Though grapes are favoured source for wine fermentation, other fruits such as apple, blueberries, mangoes and pineapple, to name a few, which contain fermentable sugars above 10%, are potential raw materials for winemaking. Additionally, distinct flavour, appealing colour and possibly can be a good source of anthocyanins/carotenoids; minerals like K, Na, Ca, Mg and Fe; and vitamins prompt wine industry to explore the possible use of a variety of fruits for wine fermentation. The natural bacterial and yeast flora on fruits, viz. lactic acid bacteria, *Saccharomyce*s and non-*Saccharomyces* yeasts, are one of the decisive factors for wine quality. More than 20 yeast genera have been reported from different fruits involved in wine fermentation. Moreover, a significant species and also strain diversity have been reported. The genetic variation in different strains leads to the expression of different biological properties which contribute to wine aroma and flavour. The knowledge of microbial diversity will possibly be useful to develop templates for quality wines. As the wine industry is now focusing on blending of different fruits with grapes for different aroma and flavour, it will be interesting to study the contribution of natural microflora of these fruits in the wine aroma and flavour, keeping intact the features of grape wine.

Keywords

Microbial flora · Grapes and other fruits · Non-*Saccharomyces* yeasts · *Saccharomyces cerevisiae* · Wine aroma and flavour

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8.1 Introduction

Grapes (*Vitis vinifera*) are commonly used for winemaking due to their high sugar contents which are adequate enough to produce a wine with more than 10% alcohol. Furthermore, acidity and different flavours, aroma and colour have made grapes as a favoured source for winemaking. Other fruits such as apple, blueberries, mangoes and pineapple, to name a few, which contain fermentable sugars with levels varying from 10 to 20%, are potential sources for winemaking. For a multi-billion dollar wine industry, two crucial aspects of quality are microbial diversity involved in wine production and chemical composition. *Terroir*, a complete natural environment in which a particular wine is produced, is also influenced by microbial diversity which usually exhibits a regional pattern.

Diverse microbial community is present on the surface of fruits, leaves, flowers and stems, as well as within their tissues. The natural microbial flora of fruits is dependent on plant species, ripening stage, soil and climatic conditions and more importantly different agricultural practices followed in the field. The interactions of microorganisms also decide the structure of microbial communities residing on the fruits. As compared to other fruits such as plums, apples, pears, cherries and strawberries, the natural flora of grapes has been studied extensively (Vepštaitė-Monstavičė et al. 2018). Most of the studies on the fruit microflora are mainly focused either on pathogens or natural antagonists that can be used to control pathogen attack.

Same grape variety grown in different regions can have wine with distinctive features which are influenced by microbial diversity. In other words, microbial biogeography is a factor which contributes to regional wine characteristics. Nevertheless, the influence of microbiota on regional wine quality is not explicitly defined. For example, the regional strains of *Saccharomyces cerevisiae* produce a distinct chemical composition of wine. Usually alcoholic fermentation is mainly carried out by *S. cerevisiae*, which leads to the formation of many alcohols and esters. Additionally non-*Saccharomyces* yeasts present also contribute in the *terroir* (Belda et al. 2017).

8.2 Quality of Fruits for Winemaking

In case of grapes, worldwide more than 10,000 varieties are used for wine production. The varieties used commonly to make red wine are: Barbera, Black Riesling, Cabernet Franc and Sauvignon, Carignan, Cinsaut, Dornfelder, Gamay, Grenache, Malbec, Merlot, Montepulciano, Muscat, Pinot Noir, Pinotage, Portugieser, Riesling, Sangiovese, Saperavi, Shiraz, Syrah, Trollinger and Zinfandel, while for white wine, Aligote, Chardonnay, Chenin Blanc, Pinot Blanc, Pinot Grigio, Sauvignon Blanc and Ugni Blanc and others are being used. The phenolic contents are more in red wine. A number of regional varieties also contribute to wine industry. The varietal character is imparted to the wine because of different secondary metabolites responsible for flavour and aroma. The stilbenes such as resveratrol, piceatannol and pterostilbene and carotenoids, beta-carotene, lutein and zeaxanthin

are present in most of the grape varieties. Grapes are a very good source of vitamin K, vitamin B2 and copper. Similar studies are relatively very less for other fruits. Joshi et al. (2017) highlighted the use of fruits other than grapes for winemaking. Different types of fruits including berries, citrus, pome fruits, stone fruits and tropical fruits can be used for wine fermentation. The basic criteria are, of course, distinct flavour, appealing colour and possibly can be a good source of anthocyanins/carotenoids; minerals like K, Na, Ca, Mg and Fe; and vitamins. In most of the fruit wines, sugar and acid contents vary. The methodologies for wine fermentation, however, could vary from source to source. Attempts are being made to commercially produce apple, apricot, banana, jamun, passion fruit, peach, plum and strawberry wines. The pulpy nature, low sugar and high acidity are main concerns. Indeed compared to grape wine, the other fruits have not so far contributed significantly to the wine industry. Reddy et al. (2014) extensively studied the wine production using different varieties of mango (*Mangifera indica*) which is helpful in reducing the postharvest losses. The six different varieties were used for wine fermentation which showed 7–8.5% (w/v) ethanol. Though other volatile compounds present in the wine were similar to grape wines, slightly higher methanol contents were reported earlier (Reddy and Reddy 2005). This was attributed to the extraction of juice using pectinases which are responsible for the hydrolysis of pectic substances to galacturonic acid and methanol. Recently, Ogodo et al. (2018) reported that mango wines have comparable sensory attributes to the wines produced using banana, pawpaw, watermelon, sweet potato and others. The analysis of aroma compounds from raspberry, strawberry and mulberry wines was reported by Feng et al. (2015). All three fruit wines prepared using commercial *S. cerevisiae* strain did not contain 4-methyl-2-pentanol, 2,3-butanediol and some alcohols which are common in grape wines. The esters were quantitatively as well as qualitatively higher in raspberry and mulberry wines than strawberry wine. In raspberry wine, 2-heptanone, 2-octanone, 2-nonanone and 2-undecanone were uniquely reported while nonanal was found to be present only in mulberry wine.

Jagtap and Bapat (2015) extensively reviewed the status and prospects of wine production from different fruits other than grapes. Depending on the nutritional composition, usually the selection of yeasts for fermentation has been suggested. For example, apple juice contains fructose, glucose and sucrose; therefore, fermentation with a fructophilic yeast strain is necessary for efficient wine production. Instead of indigenous microbial flora, the wine fermentation using commercial strains was discussed. Other than nutrition and sensory perceptions, the main concern was to minimize post-harvest losses.

8.3 Microbial Flora on Grapes and Other Fruits

The yeasts, *Saccharomyces* and non-*Saccharomyces*, and lactic acid bacteria, a natural flora on fruits, are considered as a decisive factor which influences wine aroma and quality. More than 20 fungal, particularly yeast, genera have been reported from different fruits involved in wine fermentation. There is a significant species and also

strain diversity. The genetic variations in different strains lead to the expression of different biological properties which contribute to wine quality.

There is a succession of different yeasts in wine fermentation which is due to their survival and proliferation under dynamic conditions. Pretorius et al. (1999) reported that in the initial stage of fermentation of grapes (*Vitis vinifera*), the genera such as *Kloeckera*, *Hanseniaspora* and *Candida* are predominating, followed by several species of *Metschnikowia* and *Pichia* when the ethanol rises to 3–4%. The final stages invariably dominated by the alcohol-tolerant strains of *S. cerevisiae.* Other species of genera such as *Brettanomyces, Kluyveromyces, Schizosaccharomyces, Torulaspora* and *Zygosaccharomyces* can also be observed during fermentation as well as in the resultant wine. Most of these yeasts are coming from vineyard and also from winery.

Chavan et al. (2009) studied yeast diversity of six wine grape varieties, viz. Bangalore blue, Cabernet, Chenin Blanc, Sauvignon Blanc, Shiraz and Zinfandel, which are being commonly cultivated in India for winemaking. The cultural and molecular studies identified eleven different species belonging to seven genera such as *Candida azyma*, *Candida quercitrusa*, *Debaryomyces hansenii*, *Hanseniaspora guilliermondii*, *Hanseniaspora vineae*, *Hanseniaspora uvarum*, *Issatchenkia orientalis*, *Issatchenkia terricola*, *Pichia membranifaciens*, *S. cerevisiae* and *Zygoascus steatolyticus.* Further Chavan (2014) reported *Candida diversa*, *Pichia kluveri, Pichia fermentans, Pichia manshurica* and *Torulaspora delbrueckii* from the same varieties. The predominant species found in all six varieties was *H. guilliermondii.* Interestingly, *C. azyma* closely associated with sugarcane phylloplane was reported first time from Bangalore blue and Cabernet varieties grown in different localities which were earlier under sugarcane crop (Chavan et al. 2009).

Martins et al. (2013) studied the epiphytic bacterial communities of grapevines variety Merlot and vineyard soils. Using terminal restriction fragment length polymorphism (T-RFLP) analysis, strains from six different bacterial classes, viz. alpha-, beta- and gamma-proteobacteria, actinobacteria, *Clostridia* and *Bacilli*, were identified from all the sources. As expected, maximum diversity was reported from soil followed by bark and then berries. The species from genera like *Pseudomonas, Curtobacterium* and *Bacillus* were found in all the ecosystems. *Pseudomonas* and *Bacillus* also reported to be endophytes involved in plant protection.

In addition to the natural flora of grapes, a number of winery equipments harbour different bacterial and fungal strains. Some seasonal variation in the microbial flora present on the winery equipments was reported (Bokulich et al. 2013). Members of Acetobacteriaceae and Lactobacillaceae were found on winery equipments after normal cleaning too. *S. cerevisiae* and *H. uvarum*, which were found to colonize on winery surfaces, act as a potential reservoir for further wine fermentation. Further, Bokulich et al. (2016) conducted studies to understand the relationship between grape microbiota and wine metabolomes in two neighbouring vineyards and their association with fermentation performance and in turn regional wine characteristics. They demonstrated that grape microbiota and metabolites are regionally different and microbial profile decides the metabolite composition of the finished wine and thus wine quality.

Santo et al. (2012) did the fermentation of strawberries (*Arbutus unedo*) and reported that initially non-*Saccharomyces* yeasts such as *Aureobasidium pullulans*, *Dothichiza pithyophila*, *Dioszegia zsoltii*, *H. uvarum* and yeasts belonging to the genera *Metschnikowia*, *Cryptococcus* and *Rhodotorula* were present. However as the alcohol concentration increased, the dominant genera were *S. cerevisiae* and *Pichia membranaefaciens*. *S. cerevisiae* strains also exhibited killer activity against *Zygosaccharomyces bailii*, a wine spoilage yeast.

In early 1960s, yeast flora on apple (*Malus pumila*) and in apple cider was reported by Clark et al. (1954). The common yeast genera observed on fruits were *Candida*, *Cryptococcus*, *Rhodotorula* and *Torulopsis*, and the *Candida* species were predominant. While in cider, *Debaryomyces*, *Pichia* and *Saccharomyces* were common. Cousin et al. (2017) extensively reviewed the status of microbial flora involved in apple cider preparation. *Candida sake* and *P. fermentans* were reported to be dominant species on apples. Pathogens like *Exobasidium* species were also observed. While in cider, *S. cerevisiae*, *Hanseniaspora* and *Brettanomyces/Dekkera* were found to be dominant. In the initial phase of fermentation *Saccharomyces bayanus* was dominant which was replaced by *S. cerevisiae* in the final stages, while *Hanseniaspora valbyensis* was present throughout the fermentation. The other yeasts present with minor percentage (<3.5% of total isolates) were *Candida oleophila*, *C. sake*, *C. stellate*, *C. tropicalis*, *H. uvarum*, *Kluyveromyces marxianus*, *Metschnikowia pulcherrima*, *Pichia delftensis*, *P. misumaiensis* and *P. nakasei*.

Vadkertiová et al. (2012) studied the yeast diversity present on apple, pear (*Pyrus* species) and plum (*Prunus* species) for 2 years at three different locations. *Galactomyces candidus*, *H. guilliermondii*, *H. uvarum*, *M. pulcherrima*, *P. kluyveri*, *P. kudriavzevii* and *S. cerevisiae* were the most frequently isolated species from the fruit samples, while *A. pullulans, M. pulcherrima* and *S. cerevisiae* were commonly found on fruits. Wei et al. (2017) isolated and identified 754 yeast strains from the skins of apples collected from 8 different localities from 2 ecological regions of China. Total 70 plus species were reported.

The most common species were found to be *A. pullulans* and *H. uvarum*, while *S. cerevisiae* was not observed in any tested samples. The other species reported with higher frequency from the samples were *Cryptococcus flavescens*, *P. guilliermondii*, *P. kluyveri* and *Rhodotorula glutinis*. Though some correlation with genera and localities was observed by correspondence analysis, there was no clear association per se with yeast diversity and geographical areas with different climatic conditions (Wei et al. 2017).

Satora and Tuszyński (2010) studied the natural yeast flora of blue plum fruits (*Prunus domestica*). *Aureobasidum* sp., *Kloeckera apiculata* and *S. cerevisiae* isolates from plum fruits and fermenting plum must were used to study fermentation. Though the fermentation was rapid with *S. cerevisiae*, the highest concentration of ethanol was detected after spontaneous fermentation carried out by *K. apiculata* and *S. cerevisiae*. The samples after spontaneous fermentation contained high levels of acetoin, ethyl acetate and total esters along with low levels of methanol and fusel alcohol.

In Zimbabwe, masau (*Ziziphus mauritiana*) is a wild fruit which harbours yeasts, and lactic acid bacteria (Nyanga et al. 2007). Using phenotypic analysis and the sequencing of the ITS 1 + 2 regions of the rDNA and the D1/D2 domains of the 26 s rRNA, predominantly yeast species such as *S. cerevisiae, I. orientalis, Pichia fabianii* and *A. pullulans* were reported. On unripe fruits *A. pullulans* was dominant, while in the fermented fruit pulp *S. cerevisiae* and *I. orientalis* were predominant. Among lactic acid bacteria, *Lactobacillus agilis*, *L. plantarum, L.. bifermentans, L. minor, L. divergens, L. confusus, L. hilgardii, L. fructosus, L. fermentum* and *Streptococcus* species were identified using physiological tests.

A number of fungi are present naturally on citrus (*Citrus* species) fruits, which sometimes have a great impact on the citrus industry and could also on wine production. Moreover, this fungal flora changes at different ripening stages. Zhao et al. (2017) reported that *Medicopsis* and *Colletotrichum* were dominant in the rind at the growth stage, *Colletotrichum* at the ripening stage, *Alternaria, Botrytis*, *Erythrobasidium, Fusarium* and *Strelitziana* at the storage stage; while in the flesh, *Botrytis, Cladosporium* and *Penicillium* were dominant. The population of plant pathogenic fungi, *Alternaria*, *Botrytis*, *Cladosporium*, *Erysiphe*, *Fusarium*, *Magnaporthe*, *Penicillium* and *Sclerotinia*, in the rind was more than in the flesh. While Perez et al. (2016) isolated few yeasts such as *Candida, Clavispora, Kazachstania*, *Pichia, Saccharomyces* and *Wickerhamomyces* from fruits and leaves of citrus plants (lemon, orange, tangerine and grapefruit) which showed killer property against pathogenic fungi.

The surface microflora of banana (*Musa* species) fruits was studied to understand seasonal [autumn (April), winter (August) and summer (December)] variation, if any (Postmaster et al. 1997). The peel of the mature pre-harvest stage fruits harboured more bacteria and fungi especially yeasts than the two earlier stages of fruit development. However, it was suggested that the influence of season was more pronounced than the stage of fruit development. Blomme et al. (2017) reviewed the bacterial pathogens attacking banana crop in addition to fungal pathogens such as *Mycosphaerella fijiensis* and *Fusarium oxysporum*. In case of bacteria, *Ralstonia, Xanthomonas* and *Erwinia* species were found to affect banana crop. The microbial flora associated with spontaneous fermentation of Plantains, green bananas having more starch than sugar, was studied by Kouadio et al. (2014). Though the organisms were not identified as specific genera, it was reported that yeasts, lactic acid bacteria and *Bacillus* spp. were observed throughout the fermentation at 25 ° C for 4 h.

Palm wine fermented from the sap of *Elaeis* sp. and from the sap of *Raphia* sp. grown in different regions of Nigeria was reported long back by Okafor (1972). The yeast genera *Saccharomyces, Candida* and *Endomycopsis* were commonly reported. Chilaka et al. (2010) isolated *Pichia* species, *S. cerevisiae*, *Saccharomyces paradoxus* and *Schizosaccharomyces octosporus* from palm wine. These isolates were further used singly for the fermentation of watermelon (*Citrullus lanatus*), passion fruit (*Passiflora edulis*) and pineapple (*Ananas comosus*). The alcohol concentration after 20 d of fermentation was 10–13%.

The pineapple wine fermentation was carried out using natural flora of non-*Saccharomyces* strains such as *Hanseniaspora opuntiae*, *H. uvarum* and *Meyerozyma guilliermondii* which were used to increase flavour diversity due to the species-dependent production of different concentrations of ketones, terpenes, norisoprenoids and a variety of phenols (Dellacassa et al. 2017).

For mango (*Mangifera indica*) wine production, *S. cerevisiae* is one of the preferred organisms which is commercially available (Ogodo et al. 2018; Reddy et al. 2014). However, Buenrostro-Figueroa et al. (2018) identified two native strains of *Kluyveromyces marxianus* for ethanol production in overripe mango pulp. Up to 5% (w/v) ethanol was produced at the end of fermentation. Santos et al. (2015) isolated 41yeast species from mango leaves. The most prevalently yeast species was *Meyerozyma guilliermondii.* With less frequency, *Bandoniozyma complexa*, *Candida akabanensis*, *Candida jaroonii*, *Candida orthopsilosis*, *Cryptococcus flavescens*, *Cryptococcus laurentii*, *Cyberlindnera bimundalis*, *Hanseniaspora opuntiae*, *Kwoniella heveanensis*, *Ogataea mangiferae*, *P. kluyveri*, *Pichia manshurica*, *Rhodotorula mucilaginosa*, *Sporidiobolus ruinemiae*, *Trichosporon asahii and Trichosporon loubieri* were reported. Some species such as *A. pullulans*, *Candida diversa*, *Candida homilentoma*, *C. quercitrusa* and others were isolated only once. Four isolates of *Ogataea mangiferae* and one *Ogataea philodendra,* methylotrophic yeasts, were found to be using methanol as a sole carbon source.

The mango-like fruit, Bambangan, *Mangifera pajang*, grows wild in the forest of Borneo Island. This has soft and tender texture, mango-like fragrance and sensory qualities. Chin et al. (2016) isolated a number of bacterial and yeast species during fermentation of bambangan. The main eight genera identified were bacteria *Corynebacterium*, *Lactobacillus*, *Micrococcus*, *Pediococcus* and *Staphylococcus* and yeasts *Candida*, *Kloeckera* and *Pichia*. The bacterial species *Lactobacillus plantarum* and *Pediococcus pentosaceus* were found predominantly in the initial phase of fermentation.

The fruits of the "umbu" tree, *Spondias tuberosa*, harbour diverse yeast community with dominant species *Candida sergipensis*, *Candida spandovensis* and *Candida sorbosivorans.* However, *Candida floricola* was reported to be superior for wine production. Other yeasts reported from the fruit surface were: *Cryptococcus flavus, Cryptococcus humiculus, Cryptococcus laurentii, I. occidentalis Kloeckera japonica, Kluveromyce*s *marxianus*, *P. membranifaciens* and *Trichosporon moniliforme-like* (Martins de Melo et al. 2007).

Hashem et al. (2014) isolated indigenous yeasts such as *H. guilliermondii, H. uvarum*, *H. opuntiae, P. kudriavzevii*, *I. orientalis, Wickerhamomyces anomalus, Yarrowia lipolytica* and *Zygosaccharomyces rouxii* from rotten date (*Phoenix dactylifera*) fruits. These were identified using the sequence of D1/D2 domain of the 26S rRNA gene. According to the authors, these indigenous isolates had potential for the bioethanol and single cell protein production.

8.4 Enumeration of Microbial Flora

A wide variety of both bacteria and fungi contribute to fruit yield, quality and, also in wine fermentation. In view of the presence of culturable and also unculturable microorganisms on fruits the metagenomic approach was used to study grapevine microbiome by Pinto et al. (2014). According to them, most abundant were ascomycetous fungi while in case of bacteria Proteobacteria, Firmicutes and Actinobacteria were present. The microbial communities were reported to be different depending on the vegetative phase of grapevine as well as on the agriculture practices. However, *Aureobasidium* and the bacteria from Enterobacteriaceae were so called unaffected due to these practices. Furthermore, in wine fermentation of non-sterile musts, the succession of yeasts and bacteria was reported by a number of researchers. In general, yeasts predominate during the alcoholic fermentation due to the low pH and high sugar contents, in general. The isolation of yeasts on malt extract agar and analysis of yeasts associated with grape wine using denaturing gradient gel electrophoresis (DGGE) was compared by Prakitchaiwattana et al. (2004). To determine yeast ecology per se on grapes, the PCR-DGGE technique was found to be less sensitive than the method of isolation on malt extract agar when the population was low (10^4 colony forming units/g). Nevertheless, the greater diversity was detected by molecular method than the agar method. *A pullulans* was predominantly observed on immature, mature, and both damaged and undamaged grapes. *Metschnikowia* and *Hanseniaspora* species were common on damaged grapes. Various species of *Rhodotorula*, *Rhodosporidium* and *Cryptococcus* were identified using the cultural method.

The diversity of yeast and other filamentous fungi in grape must and wine fermentation was studied extensively (Wang et al. 2015). The methods used were culture-dependent and culture-independent, and identification was carried out using 5.8S-ITS-RFLPand26S-D1/D2 sequencing. Usually a high species richness was observed in the grape must after crushing, which includes *Aspergillus tubingensis*, *A. pullulans* or *Starmerella bacillaris*. However, as fermentation proceeded, the species richness went down and the yeasts such as *H. uvarum, S. bacillaris* and *S. cerevisiae* dominated the must. The *terroir* characteristics of the fungal diversity were more related to the geographical location than the grape variety. Oliveira et al. (2017) studied epiphytic fungal community of grapevines from different regions of Portugal. Using internal transcribed spacer region, 18S rRNA and β-tubulin gene they identified genera like *Alternaria, Caldosporium, Penicillium, Aspergillus* and *Epicoccum* in a decreasing order of their abundance.

Due to *Botrytis cinerea* infection on grapes the sugar contents increase characteristically. Usually sweet white wines are made from such infected grapes (Mills et al. 2002). This infection also alters species heterogeneity and succession during the fermentation. Using both culture-dependent (plating) and culture-independent (PCR-DGGE and reverse transcription-PCR [RT-PCR]-DGGE) methods, the main yeasts reported were *Saccharomyces*, *Hanseniaspora*, *Pichia*, *Metschnikowia*, *Kluyveromyces* and *Candida* species in the fermentations carried out under different temperatures. In plating, as well as using 26S rDNA sequence analysis, six

morphotypes, viz. *S. cerevisiae*, *H. uvarum*, *P. kluyveri*, *M. pulcherrima*, *Kluyveromyces thermotolerans* and a *Candida* strain, were identified. The metabolically active *Candida* population was found to be persisted throughout the fermentation but could not be compared quantitatively by the plating method. The acetic acid bacteria in *Botrytis*-affected musts produce gluconic acid, 5-oxofructose and dihydroxyacetone which reduce the effective concentration of SO_2 which results in uncontrolled microbial growth. Nisiotou and Nychas (2007) observed higher diversity in the initial and middle stage of fermentation than their healthy counterparts which was attributed to the presence of fermentative and/or spoilage species, such as *Zygosaccharomyces bailii* and *Issatchenkia* sp. or *Kluyveromyces dobzhanskii* and *Kazachstania* species. The species diversity in both the fermentations was studied using both culture-based and -independent molecular methods.

Using high-throughput pyrosequencing of 16S rRNA gene amplicons, the bacterial diversity associated with Chardonnay, a variety of grapes was studied (Leveau and Tech 2011). The major genera observed on leaves and berries were *Achromobacter*, *Bacillus*, *Cellvibrio*, *Curtobacterium*, *Flavobacterium*, *Hymenobacter*, *Leuconostoc*, *Massilia*, *Methylobacterium*, *Pseudomonas*, *Skermanella* and *Sphingomonas.*

Wei et al. (2017) studied yeast flora from the skins of apples collected from eight different localities from two ecological regions of China. More than 71 species from 24 genera were identified following sequencing of the 26S rRNA gene (S) D1/D2 domain. The most common species were found to be *A. pullulans* and *H. uvarum.* The high percentage of *C. flavescens*, *P. guilliermondii*, *P. kluyveri* and *R. glutinis* was also reported.

Morrissey et al. (2004) identified indigenous yeasts from traditional Irish cider fermentation using PCR-RFLP analysis of ITS1, ITS2 and the 5.8S rRNA gene. The succession of different yeast species during fermentation was seen as *Kloeckera, H. uvarum* in the initial phase, then *S. cerevisiae* and finally domination by *Dekkera* and *Brettanomyces* type yeasts. Other genera reported were *Pichia* and *Saccharomycodes*. Vepštaitė-Monstavičė et al. (2018) using next-generation sequencing-based metagenomic analysis of natural flora of apple reported limited geographical differentiation of microbial flora associated with apples. The dominating group of bacteria was from Gammaproteobacteria, mostly family Enerobacteriaceae and in case of yeasts it was a class Saccharomycetes. While using similar analysis, for blackcurrent (*Ribes nigrum* L) berries harvested from different regions, they observed high microbial diversity. The genera such as *Acinetobacter*, *Flavobacterium*, *Pantonea*, *Staphylococcus*, *Streptococcus* and *Tatumella*, to name a few, showed some correlation with geographically distinct localities. The yeasts from classes Dothideomycetes and Saccharomycetes showed correlation with localities. In general, the species of beneficial genera such as *Cryptococcus*, *Hanseniaspora*, *Massilia*, *Rhodotorula* and *Sphingomonas* and phytopathogens like *Cladosporium*, *Pantoea*, *Phoma*, *Pseudomonas*, *Septoria* and *Taphrina* were reported.

8.5 Biochemical Activities of Microflora

The role of microbial diversity in vineyard and during wine fermentation has been highlighted in recent years. Though *S. cerevisiae* is dominating the fermentation, non-*Saccharomyces* yeasts are also gaining lot of importance (Liu et al. 2017). In the initial stage of wine fermentation, non-*Saccharomyces* yeasts contribute mainly to organoleptic characteristics of wine and not fermentation, per se. In this regard a wide variety of yeast species from different grape varieties were reported (Chavan et al. 2009; Mane et al. 2017). Several factors have been described as determinants of microbial diversity in enological environments. In 1960s, *Torulaspora delbruckii* was used to reduce volatile acidity. Non-*Saccharomyces* yeasts are also involved in reducing ethanol and enhancing glycerol contents and release of mannoproteins and in the modulation of wine aroma, i.e. concentration of terpenes (Belda et al. 2016). A large number of non-*Saccharomyces* yeasts belonging to genera, *Brettanomyces*, *Candida*, *Debaryomyces*, *Hanseniaspora*, *Hansenula*, *Kloeckera*, *Kluyveromyces*, *Lachancea*, *Metschnikowia*, *Pichia*, *Schizosaccharomyces*, *Torulaspora* and *Zygosaccharomyces*, produce flavor-active compounds which contribute into the overall aroma. Jolly et al. (2013) reviewed the studies on non-*Saccharomyces* organisms for their contributions, singly and in combination, during wine fermentation especially for wine aroma and flavour. The contributions will depend on the concentration of metabolites, and environmental conditions, such as osmotic pressure, relative proportion of sugars, presence of SO_2, temperature, anaerobic condition and most importantly decreasing nutrients and increasing alcohol concentration. The metabolism in these organisms leads to the formation of products such as terpenoids, esters, higher alcohols, glycerol, acetaldehyde, acetic acid and succinic acid (Table 8.1). Though the general patterns of production of metabolites involved in aroma and flavour described at a species level, the strain-dependent quantitative variations can also be seen (Belda et al. 2017; García et al. 2016). The yeasts, *S. cerevisiae* and *P. guilliermondii*, also affect formation of vitisin A and B type pro-anthocyanins with the help of 4-hydroxy-cinnamate decarboxylase (4-HCD, also called *p*-coumarate decarboxylase) activity producing highly reactive vinylphenols important in wine colour. While some yeasts were also reported to be involved in the formation of polymeric pigments (quinonoid anhydrobase chromophores derived from the anthocyanins stabilized by substitution with reactive flavans) (Morata et al. 2016).

Barbosa et al. (2015) suggested that co-inoculation of non-*Saccharomyces* yeasts and *S. cerevisiae* modulated wine aroma not only by individual metabolite production but also by changing the genomic expression patterns. For instance, the expression levels of different amino acid and ammonium transporters involved in the production of aromatic compounds, such as higher alcohols, and varietal thiols are influenced by coexistence.

Belda et al. (2017) discussed biochemical pathways involved in the production of flavour compounds and microbial complexity connecting metagenomics with metabolomics and proposed new directions involving human perceptions of wine. According to the authors, it could be possible that oral bacteria might be involved in

Table 8.1 Non-*Saccharomyces* yeast and their metabolites important in wine flavour and aroma

Yeast	Metabolites	Remarks
Candida pulcherrima	Terpenes and volatile thiols, ester ethyl octanoate	Varietal aroma of Debina wine; sequential fermentation with *S. cerevisiae* for Sauvignon Blanc and other wines
Candida zemplinina/ Candida stellata	Glycerol, succinic acid, terpenols (linalool, citronellol, geraniol, nerolidol and farnesol), ethyl esters and short-chain fatty acids	Sequential fermentation with *S. cerevisiae* for Sauvignon Blanc; while Co-inoculation/ sequential Chardonnay wine low aroma intensity
Hanseniaspora species *H. guilliermondii/H. uvarum/H. vineae*	2-Phenyl-ethyl acetate, hexyl acetate, ethyl acetate and isoamyl acetate, ethyl esters 2-(methylthio)-ethanol, 2-methyl tetra hydrothiophen-3-one, acetoin	Co- inoculation of *H. uvarum/ S. cerevisiae* for Sauvignon Blanc; sometimes undesirable biogenic amines are produced
Hansenula anomala	Higher alcohols, acetate and ethyl esters	With *S. cerevisiae* wines with decreased C6 alcohols and thioalcohol levels
Issatchenkia orientalis	Acetaldehyde, propanol, 2-butanol and isoamyl alcohol	In co-fermentation of grape juice with *S. cerevisiae* malic acid in wine is reduced
Kluyveromyces thermotolerans	Lactic acid, glycerol and 2-phenylethanol	Combination with *S. cerevisiae* enhances aroma and flavour in white and red wines
Pichia kluyveri	Thiols, especially 3-mercaptohexyl acetate, zymocins (killer toxins)	Useful in Riesling, Sauvignon Blanc and Chardonnay wines; zymocin can inhibit certain *S. cerevisiae* strains
Pichia fermentans	Acetaldehyde, ethyl acetate, 1-propanol, n-butanol, 1-hexanol, ethyl octanoate, 2,3-butanediol and glycerol	*P. fermentans/S. cerevisiae* combination increases concentration of polysaccharides
Saccharomycodes ludwigii	Higher alcohols, acetic acid, isobutanol, amyl alcohol, ethyl lactate, polysaccharides	Fermentations of Trebiano grape must in monoculture
Schizosaccharomyces species *S. Pombe/S. maldivorans*	Acetaldehyde, propanol and 2,3-butanediol and 1,1-diethoxyethane	Degrade malic acid and gluconic acid
Torulaspora delbrueckii	Succinic acid, linalool, ethyl hexanoate, ethyl octanoate phenylethyl acetate, β-phenyl ethanol and isoamyl acetate	Varietal aroma of Muskat type wine; co-inoculation with *S. cerevisiae* for Sauvignon Blanc and Chenin Blanc wines
Torulopsis cantarellii	Glycerol, acetoin, propanol and succinic acid	Syrah wines in mixed fermentation with *S. cerevisiae*

(continued)

Table 8.1 (continued)

Yeast	Metabolites	Remarks
Williopsis saturnus	Acetic acid, propanol, ethyl acetate and isoamyl acetate	Mixed fermentations of Emir grape juice with *S. cerevisiae* shows minor variation in flavour as compared to only *S. cerevisiae* monoculture wines
Zygosaccharomyces species	Acetic acid, H_2S, SO_2, polysaccharides	*Z. fermentati* produces low levels of acetic acid, H2S and SO2; *Z. bailii/S. cerevisiae* and *Z. florentinus/S. cerevisiae* have shown increased production of polysaccharides, which can have a positive influence in wine taste; *Zygosaccharomyces* species fructophilic in nature

Adapted from Belda et al. (2016, 2017); Canonico et al. (2016); García et al. (2016); Jolly et al. (2013)

conversion/release of wine aroma. Therefore, understanding of these bacteria might be useful to adjudge wine quality. Furthermore, the attempts of different researchers to develop new *S. cerevisiae* strains using synthetic genomics, if successful, can take the wine industry to a different level.

Van Rensburg and Pretorius (2000) highlighted the significance of endogenous enzymes from grapes and also from natural yeast flora in the winemaking. The enzymes such as pectinases, glucanases, xylanases and proteases are important for the clarification and processing of wines, while glycosidases contribute to release of varietal aromas from precursor compounds. The ethylcarbamate, a carcinogen is naturally formed in wine due to urea and ethanol. The urease and glucose oxidase reduce the levels of ethylcarbamate by affecting levels of urea and ethanol. The glucosidases from grapes as well as from non-*Saccharomyces* yeasts can contribute to the release of aroma compounds. However, the indigenous enzymes of grapes are not adequate in hydrolysing their own non-volatile glycosidic precursors. Possible reasons could be narrow substrate specificity, easy inactivation due to low pH (between 3.0 and 4.0) and high glucose concentration (>1%). The clarification of wine is facilitated by the enzymes like pectinases, glucanases, xylanases, lipases and proteases. As *S. cerevisiae* does not produce significant quantities of these enzymes, non-*Saccharomyces* yeasts play an important role in wine fermentation (Ganga and Martinez 2004; García et al. 2016). The predominant genera which produce these enzymes are *Brettanomyces, Candida, Cryptococcus, Debaryomyces, Hanseniaspora, Hansenula*, *Kloeckera, Kluyveromyces*, *Metschnikowia, Pichia, Rhodotorula, Saccharomycodes, Schizosaccharomyces* and *Zygosaccharomyces.* Maturano et al. (2012) studied the enzymes from *S. cerevisiae* and non-*Saccharomyces* species (*H. vinae*, *T. delbrueckii*) in pure and mixed culture during the fermentation of sterilized grape juice. In mixed cultures, though *H. vinae* and *T. delbruckii* were detected in the initial period of the fermentation the β-glucosidases, proteases and pectinases secreted by *H. vinae* and *T. delbruckii* could be detected up to the end of fermentation. Enzyme profiling was carried out during fermentation of

Shiraz and Cabernet grape varieties. Pectinase, β-1,3-glucanase and protease activities increased from 3 to 6 d, while β-glucosidase activity decreased after 9 d. These enzymes correlated significantly with secondary metabolites, such as total phenolics, flavonoids and tannins that are important to wine quality (Chavan 2014; Mane et al. 2017). *C. azyma*, *H. guilliermondii, H. uvarum, Issatchenkia terricola, Pichia* and *Torulaspora* were found to be major contributors. The commercial enzyme preparations were also tried for different functions at various stages during winemaking. For example, *Trichoderma harzianum* β-glucanases were used for clarification, filtration and maturation (Van Rensburg and Pretorius 2000). Mojsav et al. (2011) used three commercial pectinases to improve filtration rates, lees settling rates and clarity of wine of white grape cultivar, Smederevka. However, the exogenously added enzymes may not perform well under the fermentation conditions. Therefore, non-*Saccharomyces* yeasts as sources of these enzymes are important during wine fermentation. Alternately, as suggested in the next section, the expression of genes of polysaccharide degrading enzymes in *S. cerevisiae* was reported to be useful (Louw et al. 2006). From the literature, it was seen that enzyme activities were influenced by pH and temperature, presence of sugars, SO_2 and ethanol. For instance, ethanol adversely affected β-glucosidase and pectinase activities during fermentation (Maturano et al. 2012).

8.6 Genetic Improvements of Wine Yeasts for Wine Fermentation

Saccharomyces, an important genus in wine fermentation, exhibits distinctive physiological properties that are not found in other non-*Saccharomyces* yeasts. Guillamón and Barrio (2017) suggested that to understand biodiversity, population structure and evolution of wine yeasts, the study of molecular aspects of wine yeast adaptation is necessary. The molecular mechanisms involved are: single nucleotide polymorphisms (SNPs), short sequence insertions or deletions, recombination and gene conversions, short tandem duplications gene and segmental duplications, gross chromosomal rearrangements (GCRs), ploidy changes and interspecific hybridization. Interestingly, a horizontal gene transfer (HGT) of a dihydroorotate dehydrogenase from *Lactococcus lactis* to *Saccharomyces* enabled it to grow under anaerobic conditions, while HGT from *Z. bailii* and from *Torulaspora microellipsoides* were found to be useful to improve fermentation efficiency (from Guillamón and Barrio 2017).

Other than natural diversity, the genetic improvement programmes, per se, to ensure the genetic modification which does not adversely affect basic wine properties have also been implemented. A number of methodologies and approaches were reported for the improvement of the wine yeasts which include mutagenesis, protoplast fusion, intraspecific and interspecific hybridization, transformation, adaptive evolution and functional genomics.

Expression of genes of polysaccharide degrading enzymes in *S. cerevisiae* was reported to be useful for wine fermentation (Louw et al. 2006). Recombinant strains

of *S. cerevisiae* were constructed using genes such as *T. reesei XYN2* xylanase, *Butyrivibrio fibrisolvens END1* glucanase, *A. niger XYN4* endo xylanase, *Erwinia chrysanthemi* pectate lyase *PEL5,* polygalacturonase *PEHL* cloned from *Erwinia carotovora* and the polygalacturonase *PEH1* from *B. fibriosolvens* (from Louw 2010). For the stabilization against protein haze, a recombinant *S. cerevisiae* strain was developed which showed improved mannoprotein-related properties (Gonzalez-Ramos et al. 2008). It was reported that the deletion of killer toxin-resistant gene (*KNR4, k*iller *n*ine *r*esistant; also known as *SMI1*) led to increased mannoprotein release in *S. cerevisiae* strains.

In the fermentation, one of the major products is glycerol produced by *S. cerevisiae* which is important in the sensory character of wine. The glycerol 3-phosphate dehydrogenase gene (*GPD2*) overexpression diverts ethanol production to glycerol which avoids the masking of regional wine flavour by higher concentration of ethanol. This strain also decreased acetic acid production. On the other hand, deletion of the aldehyde dehydrogenase gene, *ALD6*, in *S. cerevisiae* also decreased acetic acid. Thus modification of *GPD2* and *ALD6* expression could be an effective strategy to increase the glycerol and decrease the ethanol concentration; this could also be useful to produce novel flavour diversity (Eglinton et al. 2002).

Srisamatthakarn (2011) studied the potential of different yeast strains in improving varietal aroma in grapes and yellow passion fruit wines. Certain commercial *S. cerevisiae* strains were reported to minimize the formation of SO_2-binding and undesirable volatile sulphur compounds and improved desirable aroma in yellow passion fruit wine.

Byaruagaba-Bazirake et al. (2013) constructed recombinant wine yeast strains with genes for glucanase, xylanase, pectinase and amylase, which yielded higher wine concentrations from the fermentation of banana pulp than the parent strain. The recombinant strains for glucanase and xylanase gave higher yields than other strains.

8.7 Future of Yeasts in Fruit Wines

Fleet (2008) extensively reviewed the importance of yeast, ecology and physiology in determining wine quality. In this regard, criteria for selecting and developing new commercial strains, the use of non-*Saccharomyces* yeasts singly or in combination, and other fermentation aspects such as membrane bioreactor for continuous fermentation were critically evaluated.

Mendes et al. (2013) studied 172 strains of *S. cerevisiae* for 30 physiological traits important in winemaking. For instance, growth in the presence of potassium bisulphite, growth at 40 ° C, and resistance to ethanol are mostly contributing to strain variability, as shown by the principal component analysis. While Naive Bayesian classifier identified resistance to antifungal compounds iprodion and cycloheximide, and growth in the presence of potassium bisulphite as important tests to identify commercial strains. Non-*Saccharomyces* yeasts contribute in almost all fruit wine quality. Therefore, the microbial profiling of wine, the profiling of

enzymes produced by these yeasts and finally profiling of aroma and flavour compounds will possibly be useful as a template for quality wine. The winemaking industry is now focusing on blending of different fruits with grapes for different aromas and flavour (https://winemakermag.com/technique/311-fruit-and-grape-blends-tips-from-the-pros). Some of the blends which have attracted consumers are grapes with blackberry, cherry, apple and mango. It will be interesting to study the contribution of natural microflora of these fruits in the wine aroma and flavour, keeping intact the features of grape wine.

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9 Harnessing Entomopathogenic Fungi for Enhanced Farm Productivity and Profitability

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Abstract

Entomopathogenic fungi form an essential component of the integrated insect-pest management strategies. The insect-pests and their entomopathogenic fungi have co-evolved over centuries and thus established an equilibrium. Species of *Metarhizium*, *Lecanicillium*, *Nomuraea*, *Isaria*, etc. have been studied extensively, and the commercial products based on *Nomuraea rileyi*, *Lecanicillium (Verticillium) leccanii*, *Beauveria bassiana* and *Metarhizium anisopliae* are popular among farmers. Several modes of action of these fungi against target pests have been elucidated. Introduction of pesticides has not only undermined the value of these fungi but also disturbed the equilibrium. The entomopathogenic fungi are living organisms formulated and delivered to the farming community for the management of target insect-pests. Due to the lag in realizing the benefit of these biocontrol agents, the farmers started using the pesticides which show their effect immediately. While on one side, indiscriminate use of these pesticides in the last three decades has led to the loss of biodiversity of beneficial organisms, pollinators, other animal and bird species; on the other side, emphasis on organic agriculture has compounded the value of these products. Thus, the interest in

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these biocontrol agents has re-invigorated. Several commercial formulations are already used by the farming community for the management of insect-pests. In this review, an attempt was made to appraise the status of these entomopathogenic fungi, their commercial exploitation, research gaps and way forward.

Keywords
Insect-pests · Entomopathogenic fungi · *Beauveria* · *Metarhizium* · *Nomuraea* · *Lecanicillium* · Biointensive insect-pest management

9.1 Introduction

The present-day commercial agricultural systems are more prone to insect-pest outbreaks as compared to the conventional agricultural systems. Over decades, in conventional agricultural systems, an equilibrium was established between crops and their major pests through a natural evolution process. This equilibrium also includes natural enemies which were parasitizing the insect-pests and thereby helping in reducing the crop losses. However, increasing pressure on food production and productivity demanded increased agricultural output leading to distortion of this equilibrium. Hence, the global agriculture started experiencing the pest outbreaks leading to major economic losses. On the other hand, indiscriminate use of pesticides and fertilizers has been affecting all biological systems.

Across global agricultural systems, the interactions of crops, insect-pests and their parasites and predators are very complex and involve multi-trophic interactions. Hence, it becomes often difficult to establish a one-to-one relationship between two interacting organisms. The in vitro studies showing a clear relationship between a given crop and a pest often fail to show similar trends under field conditions where the interacting elements increase. These interactions are also significantly influenced by physical weather parameters which add a new dimension to the complexity.

World over, major crop losses due to insect-pests have been extensive affecting food and nutritional security. Both in developed and developing countries the damage by insect-pests has been substantial. While the lepidopteran pests are known to cause damage to plant parts by feeding on them, the sucking pests cause not only physical damage to plants but may also transmit virus diseases as vectors. The mean avoidable yield losses due to insect-pests on castor cultivars were higher (22.5–89.4%) during *rabi* season compared to 17.2–63.3% during *kharif* season (Lakshminarayana and And Duraimurugan 2014). However, Dhaliwal et al. (2015) reported that economic losses due to insect-pests have reduced from 13.6% to 10.8% at global level and in India; the losses have declined from 23.3% to 15.7%. Cerda et al. (2017) assessed primary and secondary yield losses in coffee in Costa Rica and reported that pests and diseases accounted for 26% primary yield losses and even higher secondary yield losses (38%). Muralidharan and Pasalu (2006) projected damage due to yellow stem borer *Scirpophaga incertulas* over rice

ecosystems up to 6.4% yield loss due to 1% dead hearts. Pod borer in pigeon pea caused yield loss of 14–100%, and the estimated values of per cent pod damage by this pest and productivity of pigeon pea have been assessed as 1137 kg ha^{-1} (Nath et al. 1977; Kumari et al. 2013). Among oilseed crops, capitulum borer *Helicoverpa armigera* in sunflower caused 30–60% damage (Dhaliwal and Arora 1994). In castor crop, *Achaea janata* in castor caused the damage from 30% to 50% while *Spodoptera litura* inflicted 25–40% loss (Srinivasa Rao et al. 2009). In tomato and brinjal, fruit borers viz. *Helicoverpa armigera* and *Spodoptera litura* caused 15–46% yield loss (Singh 1997; Kulkarni and Shekarappa 2001). Babu and Azam (1989) reported that grape mealybugs could cause 90% yield loss.

Microbial pesticides have been found to be effective in managing insect-pests. These essentially consist of bacteria, entomopathogenic fungi or viruses and also include the metabolic products of bacteria or fungi. Some of the commercialized microbial pesticides include *Nomuraea rileyi, Verticillium leccanii*, *Beauveria bassiana* and *Metarhizium anisopliae*. There are more than 700 fungal species from at least 90 genera which are known to be effective against insects (Khachatourians and Sohail 2008). They attack a wide range of insect and mite species, but some fungal species and strains are very specific. Entomopathogenic fungi belong to the families of Zygomycota and Ascomycota and in the class of Hyphomycetes in Deuteromycota (Samson et al. 1988) as well as in the families of Chytridiomycota and Oomycota. Though many fungal taxa contain entomopathogenic fungi, most popular ones are placed in Hyphomycetes (*Beauveria bassiana*, *Verticillium lecanii, Metarhizium anisopliae* and *Nomuraea rileyi) and* Zygomycetes (*Entomophthora muscae; Erynia* [*Zoophthora*] *radicans)*. While host range of some entomopathogens is restricted to a single family (e.g. *N. rileyi* on Noctuidae) or a single order (*E. muscae* on Diptera), some may show a wide host range including several orders (e.g. *B. bassiana* on *Lepidoptera*, Hemiptera and Coleoptera). The diversity, multiplication, applications and commercialization aspects of some of the common entomopathogenic fungi are discussed below.

9.2 Diversity of Entomopathogenic Fungi

Among the microbial biological control agents, fungal pathogens have received particular interest because of their effective management of pests with piercing and sucking mouthparts (Wraight et al. 2001) compared to entomopathogens like bacteria or viruses which infect through gut wall, and these have been ingested by insect-pests which are difficult for the insects with piercing and sucking mouthparts. In addition to this, almost all insect orders are susceptible to fungal diseases. The added advantage of entomopathogenic fungi over other microbial control agents is that they are capable of attacking each and every stage of the developing insect including pupal stages (Ferron 1978 and Anand et al. 2009). Most entomopathogenic fungal isolates are specific to the insect taxons or to closely related species. Out of the 750 species of entomopathogenic fungi known at present, only 10 species are presently being developed for biocontrol of insect-pests. *Beauveria*, *Metarhizium*,

Lecanicillium, *Isaria*, *Hirsutella*, *Nomuraea rileyi* and Entomophthorales are the important anamorphic genera of entomopathogenic fungi. Species from all these genera are used as biopesticides for the control of insect-pests.

9.2.1 Beauveria bassiana

Beauveria bassiana (Hyphomycetes) is an entomopathogenic fungus (parasitic to insects), which grows naturally in soils throughout the world. It was earlier known as *Tritirachium shiotae*. It is pathogenic to a wide variety of arthropods, including thrips, whiteflies, aphids, termites, ants, beetles, weevils, grasshoppers, mealybugs, bedbugs caterpillars and vectors of malaria-transmitting disease. It infects a wide range of immature stages of insects as well as adult insects and is available commercially as a microbial insecticide. It causes a disease in insects called as the white muscardine disease. In 1835, an Italian entomologist, Agostino Bassi, first observed *Beauveria bassiana* as the cause of the muscardine disease of the domesticated silkworms. Hence, *Beauveria bassiana* was named after him. More than 700 species are reported to be the natural host of *B. bassiana* (Inglis et al. 2001). It temperate regions almost all major insect taxa are known to be the most common natural host for this fungi. Susceptibility of insects to different strains of this fungi varies considerably, and hence strains have been collected from different infected insects, and their efficacy is evaluated through in vitro and in vivo studies.

9.2.2 Metarhizium anisopliae

Metarhizium anisopliae, earlier called as *Entomophthora anisopliae*, is a soil-inhabiting fungus distributed all over the world. For the first time in 1879, Elie Metchnikoff used *M. anisopliae* as a microbial agent against insects. He used it in experimental tests for the management of wheat grain beetle, *Anisoplia austriaca*. Later it was used to control the sugar beet curculio, *Cleonus punctiventris*. *M. anisopliae* belongs to the phylum Ascomycota and a member of the Hyphomycetes class of fungi. It is an anamorphic fungus and it is categorized as a green muscardine fungus due to the green colour of the sporulating colonies. It is pathogenic to more than 200 species of insects and other arthropods. Among the genus *Metarhizium, M. anisopliae* is the most intensively studied species.

The reproductive structures of *M. anisopliae* comprise conidiophores and conidia. It produces leveduriform structures or blastospores and appressoria through mycelial differentiation. In certain cases blastospores can function as reproductive units, and these are produced in submerged cultures (Jackson and Jaronski 2009) and in the haemolymph of host insects (Alves 1998). The hyphae, produces appressoria, at the extremity is involved in fungus pathogenicity and initiates epicuticular and procuticle penetration of the insect integument (Alves 1998). It is considered as a very effective microbial control agent for the management of ticks and Lyme disease as it is highly pathogenic to many species of ticks. *M. anisopliae* is applied to

wood containing active termite galleries. Termites in these galleries come in direct contact with the fungus. In addition to direct contact with the fungus, infection of other termites in the colony takes place when grooming individuals exposed to the fungus spread the fungus to healthy, non-infected termites in the population. Depending on temperature, death of the termites occurs within 4–10 days.

9.2.3 Nomuraea rileyi

Entomogenous fungus *Nomuraea rileyi* is a dimorphic hypomycete, which is capable of causing fungal epizootics in many noctuid insect-pests (Vargas et al. 2003). It is present throughout the world infecting noctuids such as *Spodoptera litura*, *Helicoverpa armigera*, *Trichoplusia ni*, *Anticarsia gemmatalis* and *Pseudoplusia includens* (Vimala Devi 1994; Shanthakumar et al. 2010). However, its pathogenicity is limited to Lepidoptera and two species of Coleoptera. The growth of the fungus starts as yeast-like bussing from germ tube and progresses to produce hypha which is cream-coloured with sticky growth. The sporulation starts from focal points and then spreads over the entire mat. Finally, the colony turns to pale green to malachite green. The hyphae are smooth and septate with a diameter of 2–3 μm. The conidia are ellipsoid, smooth, pale green, born in divergent chains and measure 3.4–4.5 × 2.0 to 3.1 μm. Usually, fungal isolates from different geographic regions vary in their pathogenicity against their host species. However, in their studies, Padanad and Krishnaraj (2009) found that 10 isolates of *N. rileyi* isolated from different parts of Karnataka, India, showed similar levels of pathogenicity against *Spodoptera litura*.

9.2.4 Lecanicillium lecanii

Zare and Gams (2001) reclassified *Verticillium lecanii* as *Lecanicillium lecanii* which is an important pathogen to insect-pests such as coccids, whiteflies and aphids (Cortez-Madrigal et al. 2003; Liu et al. 2011). It forms a characteristic white mycelial growth on the edges of infected scale insects; hence it is known by name “white halo fungus”, Viegas first reported in 1939, who referred to the characteristic white halo formed by the fungus on the scale insect *Coccus viridis* (Green) as “the farmers friend”. Throughout the world, it is one of the most important and commonly found entomophagous Hyphomycetes fungi which occurs on Diptera, Hymenoptera, Lepidoptera and sucking pests such as coccids, aphids, thrips and mites. This fungus is also effective to several species of Nematodes (Schuler 1991). Similar to other entomopathogenic fungi, it infects its invertebrate hosts through the external cuticle. Three phases have been recognized in the development of insect mycosis: adhesion and germination of the fungal spores on the host cuticle, penetration of the insect integument by a germ tube and development of the fungus inside the insect body, generally resulting in the death of the infected host. The dead host

is covered by the fungal spores and hyphae in high humid conditions (Quinlan 1988; Zimmermann 1986).

9.2.5 *Isaria* spp.

For more than 30 years, Entomopathogenic *Isaria farinosa* and *Isaria fumosorosea* were called as *Paecilomyces farinosus* and *Paecilomyces fumosoroseus* (Wize) Brown and Smith (Hyphomycetes). Both fungi are found throughout the world and possess a broad host range. Presently, *I. farinose* is of less importance in research and as a biocontrol agent, whereas *I. fumosorosea* is considered as a species complex, and different strains are successfully used for control of whiteflies and several insect-pests (Gisbert Zimmermann 2008). *I. fumosorosea* causes the disease known as yellow muscardine, and it is one of the most important natural biocontrol agents of whiteflies (Nunez et al. 2008). Both in greenhouse and open field environments, it is known to cause strong epizootics against whitefly species such as *Bemisia* and *Trialeurodes*. *P. fumosoroseus* is effectively used for the control of nymphs of whitefly (Kim et al. 2002) and mosquito sp. *Culex pipiens* (Sandhu and Mishra 1994). This fungus has a wide host range and infects more than 40 insect species spread over 25 different families and many species of mites. Agricultural insect-pests such as the Russian wheat aphid, diamondback moth and the silver leaf whitefly are susceptible to its infection. Among mites, the European red mite, spotted spider mite, brown mite and apple rust mite are the susceptible species and it produces blastospores in liquid medium and conidia on solid media. Since two types of propagules are produced by the fungus, it is essential to study the efficacy of the propagule against target insect-pest and feasibility of mass multiplication of the same to determine its fitness for commercialization. Such situation is often encountered in many entomopathogenic fungi.

9.2.6 Hirsutella spp.

Among Deuteromycetes, Hirsutella sp. infections are known from eriophyids. The genus Hirsutella consists of more than 50 species of entomogenous fungi, but only a few have been reported as pathogenic to eriophyids. Hirsutella *thompsonii*, Hirsutella *gigantea* and Hirsutella *citriformis* are the three important species of the genus Hirsutella. Among all major genera of fungal entomopathogens, the genus Hirsutella has been one of the most difficult members for identification largely because of the large number of species and high variability among these species. Hirsutella *thompsonii* is used for the control of citrus rust mite and it is also pathogenic to Acarida, Lepidoptera and Hemiptera. The citrus rust mite, *Phyllocoptruta oleivorus,* was found to be most susceptible on grapefruit, going from 5000 mites on a single grapefruit to sudden decimation of its populations (reduced to almost zero) due to Hirsutella *thompsonii*.

9.2.7 Entomophthorales

The order Entomophthorales contain many genera which are obligate entomopathogenic species with limited host range. Under natural conditions, they are capable of producing epizootics in insect populations. Among entomopathogenic fungi, entomophthoralean fungi (Zygomycota: Zygomycetes) are considered as unique group which are obligate entomopathogens of aphids and cause natural epizootics among aphid populations (Hatting et al. 1999). Species of entomophthoralean fungi that are isolated and identified from aphids belong to eight genera: *Conidiobolus*, *Entomophthora*, *Erynia*, *Neozygites*, *Batkoa*, *Pandora*, *Tarichium* and *Zoophthora* (Bałazy 1993; Keller 1987, 1991). Six species of Entomophthorales, viz. *Neozygites fresenii*, *Zoophthora radicans*, *Entomophthora planchoniana* Cornu, *Conidiobolus obscurus, Pandora neoaphidis* (Remaudière & Hennebert) Humber, *Entomophthora planchoniana, Zoophthora* sp. and *Conidiobolus obscurus,* were found to infect aphids are common biocontrol agents of aphids, which lack epizootic potential. *Neozygites fresenii* can cause epizootics in dense aphid colonies, mainly in those of the black bean aphid.

Neozygites species were reported to cause 32–95% adult mortality in *Panonychus citri*, the citrus red mite. Several other species of spider mites such as *Eutetranychus banksi* and *Tetranychus urticae* were found to be susceptible to *Neozygites* sp. *Neozygites fresenii* (Nowakowski) Batko (Entomophthorales: Neozygitaceae) is an insect pathogenic species which is capable of causing natural epizootics in aphid populations on cotton. Epizootics caused by *N. fresenii* have been reported in the United States and in Africa. Epizootics of *N. fresenii* were observed in *Aphis fabae* Scopoli (Homoptera: Aphididae) infecting sugar beets (Gustafsson 1969). The entomopathogenic fungi, *Neozygites floridana* Weiser and Muma (Mietkiewski et al. 1993) and *Neozygites adjarica* (Dick and Buschman 1995), are used for the management of plant-feeding mites. Among mite stages, immature stages and females of *T. urticae* are more susceptible to *N. floridana* infection compared to males.

Entomophthora spp. are obligate parasites which require a live host to survive, and mass production of this group of fungi is complicated by the fact that they require a live host and they are highly specific. Natural epizootics of entomophthoralean fungi such as *Neozygites fresenii* and *Pandora neoaphidis* (in aphids), *Entomophaga maimaiga* (in gypsy moth), *N. floridana* (in mites) and *Entomophthora muscae* (in flies) are capable of causing significant reductions in host populations. These fastidious fungi are still considered as important natural control agents of some pest species, although these fungi are difficult to mass multiply in artificial media and they do not have the potential to be sold as biopesticides.

9.2.8 Other Minor Entomopathogenic Fungi

Beauveria brongniartii (Saccardo) Petch [Deuteromycotina, Hyphomycetes] is an insect pathogenic fungus, considered the most important pathogen effective against European cockchafer, *Melolontha melolontha* L. (Coleoptera: Scarabaeidae) (Keller

1997). It is commercially used as a microbial control agent for the management of larvae of *M. melolontha* in grasslands and orchards. *Metarhizium acridum* (Bischoff et al. 2009) is a group of fungal isolates that are known to be virulent and specific to acridid insects (grasshoppers and locusts). *M. flavoviride* is known to be virulent against hemipterans and some coleopteran insect species. Studies have shown that Met52®, which contains the entomopathogenic fungus *Metarhizium brunneum*, is effective for the control of *Ixodes scapularis*, the tick vector for the bacterium causing Lyme disease and tick-vectors. The entomopathogenic genus *Aschersonia* (Deuteromycotina: Hyphomycetes) is host specific to some whiteflies and scale insects. *Aschersonia aleyrodis* was the first fungal pathogen used for the successful management of citrus whiteflies in North America (Liu et al. 2006). In the tropics and subtropics, *A. placenta* Berkeley and Broom has been considered as an important entomopathogenic fungus which has potential to cause epizootics in whiteflies and scale insects (Fransen 1990; Zhu et al. 2008).

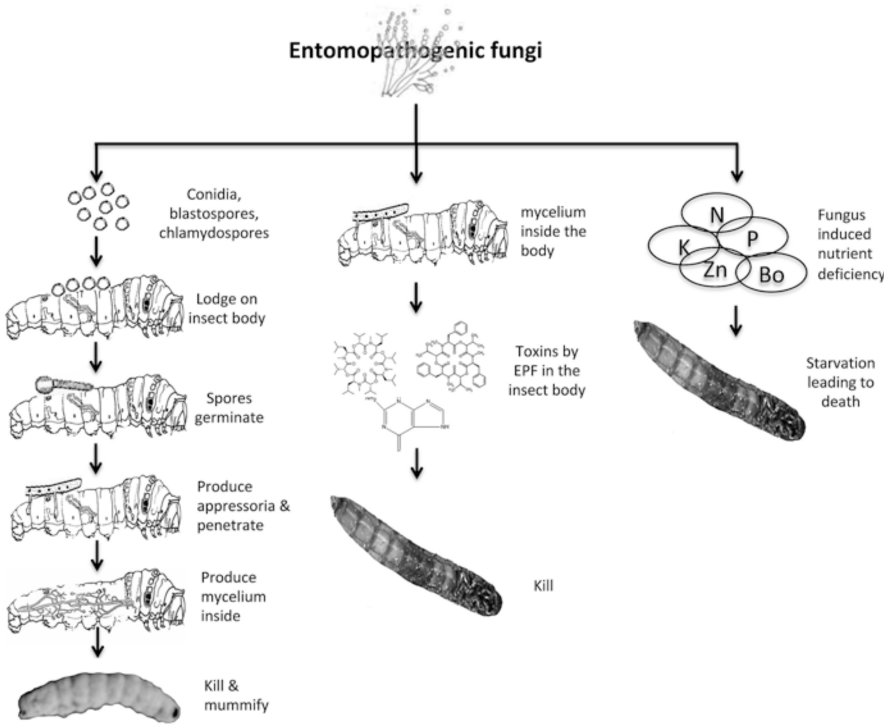

Fig. 9.1 Different modes of action of entomopathogenic fungi on insect-pests

9.3 Mode of Action of Entomopathogenic Fungi on Insect-Pests

Entomopathogenic fungi adopt several modes of action against their target hosts. The fungi are known to produce a battery of hydrolytic enzymes and toxins. These enzymes and toxins attack the insect-pests and cause death. A generic mode of action of entomopathogenic fungi is shown in Fig 9.1.

9.3.1 B. bassiana

When fungal spores adhere to the body of insect hosts, by a combination of physical force and enzymatic action the spores germinate on the cuticle and penetrate the insect body. There, the fungus multiplies inside the insect's body, producing toxins and draining the insect of nutrients, leading to the death of the insect. When the fungus has killed its host, it grows back out through softer areas of the cuticle while covering the whole insect body with white mould. Millions of new infective spores are produced by this white mould which are released into the environment. The death of infected insect host occurs in 3–5 days. Dissemination of the fungus takes place by the dead insect which contains millions of spores. The fungus is also transmitted by an infected adult male through mating (Long et al. 2000). *Leptinotarsa decemlineata*, Colorado potato beetle, and *Halyomorpha halys*, brown marmorated stink bug are infected by the pathogenic fungus, *B. bassiana*. Application of *B. bassiana* resulted in 93% reduction in the populations of *Tetranychus urticae*, two spotted spider mite in greenhouse tomato (Chandler et al. 2005) and about 60–86% reduction on different vegetables (Gatarayiha et al. 2000). Dara (2015) showed that in organic celery, application of combination of *B. bassiana* and azadirachtin resulted in 62% reduction in the population of *Rhopalosiphum rufiabdominale*, rice root aphid, and *Hyadaphis foeniculi*, honeysuckle aphid.

9.3.2 Metarhizium anisopliae

Through the spiracles and pores in the sense organs the pathogenic fungus, *M. anisopliae* makes entry into the insect body. Inside the insect body, it produces a lateral extension of hyphae, which eventually proliferate and internal contents of the insect body are consumed. The fungus continues to grow the hyphal extensions till the insect body is completely filled with mycelia. The fungus consumes the internal contents of the insect host and then it breaks through the cuticle and sporulation takes place, which makes the insect appear "fuzzy". The fungus also obtains nutrition from the lipids present in the cuticle. Secondary metabolites, such as destruxin are produced by the fungus, which are toxic to moth and larvae of flies. The relationship between the fungus and insect is established by the adhesion and germination of conidia on the surface of the insect body, followed by penetration of hyphae through the cuticle. After penetration, the process of host colonization is initiated,

as the penetrating hyphae become thicker and ramify within the tegument and haemocoel of the insect, forming blastospores. The hyphae continue to grow and invade various internal organs of the insect body. Once the host insect is dead, the fungus emerges from the insect body and produces conidia that serve as secondary source of infection to other individuals of the insect-pest populations (Alves 1998; Sandhu et al. 2012).

9.3.3 Nomuraea rileyi

The infection process of *N. rileyi* begins when conidia comes in contact with the insect cuticle (Srisukchayakul et al. 2005). The germ tube enters through the cuticle, which causes lysis of endocuticle; subsequently, the hyphal bodies are developed in the haemocoel which convert to invasive mycelia leading to death of the host insect. At the end of the infection cycle, mycelia comes out from the cuticle and produces conidiophores (Srisukchayakul et al. 2005). It also produces a proteinaceous substance which inhibits the metamorphosis and moulting of larvae (Kiuchi et al. 2003). After reaching the haemocoel, the fungus multiplies to produce hyphal structures and spreads locally inside the insect body. Within a short time, the fungus completely covers the larval body and starts sporulation. Under congenial environmental conditions, the fungus kills the larva and sporulates intensely. Interestingly, the fungus can infest its host from outside itself whereas, in case of bacteria and viruses, which requires to be ingested by the insect.

9.3.4 Lecanicillium lecanii

When conidia come in contact with the host integument, it gets adhered to the epicuticle and germinate and the germ tubes formed from germinated conidia penetrate cuticle directly or grow over the surface of the epicuticle (et al. 2012). The germ tube penetrates by lysing both epicuticle and procuticle. The fungus produces a number of extracellular enzymes which play an important role during cuticle penetration of insect host. It produces different extracellular enzymes like proteases, chitinases (St. Leger et al. 1986; St. Leger et al. 1987), esterase, N-acetylglucosamine, endoprotease, chitinase, aminopeptidase, carboxypeptidase A, lipase and Pr1-chymoelastase serine protease (Goettel et al. 1989). All these enzymes serve as cuticle-degrading enzymes. Among these enzymes, Pr-1 serves as major cuticle degrading enzyme as its concentration increased at the site of penetration peg in comparison to other enzymes. An active digestion and absorption of cuticular components accesses, colonizing hosts tissues and producing elongated hyphal bodies (blasterspores). The pathogen causes infection symptom of colour change of aphid from original yellow to red which turns to dark brown before the visible fungal growth over the body surface of *Aphis gossypii* Glover (Shinde et al. 2010). An insect dies due to mechanical pressure exerted by excessive fungal growth and action of mycotoxins (Ferron 1981). The mycotoxins produced by *L. lecanii* are

namely, beauvericin, bassianolide (Kanaoka 1978; Suzuki et al. 1977), dipicolinic acid (Claydon and Grove 1982) vertilecanin-A1, decenedioic acid and 10-hydroxy-8-decenoic acid (Soman et al. 2001). As the host nutrients are depleted, the blastospores differentiate into elongated hyphae which extend outward from the body forming a mycelial mat of conidiophores over the surface of the integument resulting in mummification (Yeo 2000). Under proper environmental condition, conidiophores mature giving rise to conidia which continues the disease cycle further (Roberts 1989).

9.3.5 *Isaria* spp.

When a spore of *I. fumosorosea* comes in contact with a suitable host, it enters into the insect's cuticle by enzymatic action. The fungus proliferates inside the insect's body by the growth of germ tube into the haemocoel. In addition, the fungus can also enter through the spiracles and other openings such as mouth or the anal. The mycelia spread in the haemolymph and other tissues of the host, eventually emerging out of the insect body and producing conidia. The death of the insect is caused by the drainage of its nutrients, the destruction of its tissues and the release of toxins by the fungus.

9.3.6 *Hirsutella* spp.

Hirsutella spp. infection starts due to penetration of the fungus through the insect host cuticle by both combination of enzymatic and mechanical processes. The spore coat possesses a mucous material which helps the spores to adhere to the host cuticle. In eriophids, the conidia germinate and invade all body parts, whereas, in case of spider mites, the penetration takes place through the legs. Hirsutellin A protein arrests the growth of the cells leading to hypertrophied condition and thus disrupting the functions of internal organelles and membranes.

9.3.7 Entomophthorales

The entomophthoralean fungi, with chitinous walls resembling hyphal bodies, enter host insect body by penetration and utilization of host nutrients. The death of the host insect occurs 3–7 days after infection due to physiological starvation. Initially round-shaped protoplasts are formed in some entomophthoralean species that either lack sugar residues in the outer cell layers or veil their presence to evade recognition by insect haemocytes (Samson et al. 1988; Glare and Milner 1991; Pell et al. 2001). The fungus emerges from the dead host, after the death of the insect and sporulation occurs outside the insect skeleton. Internal sporulation of the fungus is observed when ambient humidity is not congenial for external sporulation. From the

attachment structures, it could be deduced that the fungus continues to stay in the new hosts for subsequent transmission.

9.4 Commercialization of Entomopathogenic Fungi

Deploying only one method of insect-pest management may not be sustainable and could lead to resurgence of the pest and also development of resistance against that strategy.

Integrated Pest Management (IPM) is a concept of deploying more than one effective pest management strategies and is considered as an effective and eco-friendly approach. The combination derived will depend on the crop-pest scenario for a given cropping system in an agroecology. Often, the modules are location/region specific and emphasize on use of minimum external inputs for optimized management of the given pest. To accomplish this objective, it is mandatory to use relevant comprehensive life cycle information of the pests on the crop for a cropping system and their interaction with the environment. The focus is also on reducing cost of cultivation while managing the pest as well as maintaining clean environment.

Biointensive IPM (BIPM) is described as "A systems approach to pest management which depends on understanding of pest bionomics and it involves accurate diagnosis of source and kind of pest problems so that a range of preventive measures and biocontrol agents could be deployed to keep pest populations within acceptable limits" (Reddy 2012). If other tactics have not been effective in the management of pests, as a last resort, chemical pesticides which are less hazardous are used with care to minimize risks. BIPM takes into consideration both ecological and economic parameters in a systems approach to manage insect-pests on a sustainable mode. While proactive BIPM relies on crop husbandry practices, reactive BIPM includes inundative releases of biological control agents among other methods. In BIPM strategies, entomopathogenic fungi have been extensively used due to their promising field performance.

9.4.1 Mass Multiplication and Field Evaluation of Insect Pathogenic Fungi

The major concern to minimize chemical pesticide usage has triggered momentum for the development of novel, eco-friendly and sustainable pest management strategies at global level. Most of the chemical pesticides have been shown to cause human health problems, environmental pollution, effects on beneficial organisms and/or development of insect resistance. Hence, biological control agents such as entomopathogenic fungi (EPF) can be potential proponents of IPM and BIPM modules as they are derived from natural ecological processes. Under natural conditions, these EPF are often known to infest insect populations and cause mortality populations. A number of fungal species such as *Metarhizium anisopliae*, *Verticillium*

lecanii and *Beauveria bassiana* are being used as biological control agents for a management of many insect-pests of crops (Tanda and Kaya 1993).

During the last few decades the use of insect pathogenic fungi as biological control agents for the management of insect species has increased throughout the world. Commercial formulations of *Beauveria bassiana* (Babu et al. 2001; Sharma 2004), *Paecilomyces fumosoroseus* (Alter and Vandenberg 2000; Avery et al. 2004) and *Verticillium lecanii* (Butt et al. 2001) are popular for management of various insect-pests. One of the essential components of the biocontrol program is the production of adequate quantities of good quality inoculums. Development of entomopathogens involves production of laboratory scale inoculum and field-testing followed by customized production system for large-scale multiplication and distribution to meet market demands. In addition, mass production of insect pathogens is cheap, they are easy to store and they are capable of withstanding a wide range of temperatures and humidity conditions. It also provides a rapid kill at economical doses, and recently, the fungus has commercially been widely developed as a microbial agent for pest management (Faria and Wraight 2001), and encouraging results were obtained against whiteflies, aphids, thrips and mealybugs in greenhouses and nurseries (Faria and Wraight 2007).

Usually, for production of blastospores, which are short lived and hydrophilic, simple and reliable production system submerged liquid fermentation multiplication procedures are followed (Romback 1989) while solid-state fermentation (Rousson et al. 1983) is adopted for the production of aerial conidia. However, a diphasic strategy is adopted in most viable mass production technologies by initial inoculum production in liquid culture which is used for further inoculating the solid substrate(s) for conidia production (Burges and Hussey 1981).

For the cultivation of microorganisms using any mass multiplication technique, the knowledge of nutritional requirements is essential. Elements such as carbon, hydrogen, nitrogen, sulphur and phosphorus are the building blocks for carbohydrates, proteins, lipids, and nucleic acids, and these play significant role in host-pathogen interaction and self-defence mechanisms. The major component is carbon and the molecules of carbon also contribute to oxygen and hydrogen. Studies indicated that growth and development of microorganisms depends on nutrient sources. Mycelia growth and spore yield largely depends on the type fungus which is used and the media components.

Macro elements such as carbon, hydrogen, oxygen, sulphur, phosphorus and nitrogen which are the components of carbohydrates, nucleic acids and proteins are required for the complete growth of microorganisms. In the tolerance selection studies, the growth characteristics along with the growth substances are useful. The solid or liquid form preparation can be used for the growth, storage and transport of microorganism (Jenkins 1995). For the full growth of microorganisms, the media should have all the nutritional requirements. Kumar and Mukerji (1996) studied several nutritional requirements for the mass production of *B. bassiana*, *M. anisopliae* and *I. fumosorosea* along with the sporulation of these filamentous fungi. In Colombia, mass production of *B. bassiana* spores is done by a simple sterilization technique based on cooked rice placed inside bottles. These *B. bassiana* spores are

mostly used for field spray applications for biocontrol of coffee berry borer (Bustillo and Posada 1996). Harvesting of spores is done by washing them out from the rice media with a one per cent oil-water suspension (Antía et al. 1992). Soon after preparation, this aqueous spore's suspension must be used immediately to prevent spore germination. Moreover, spore longevity is reduced if they are kept in the bottles because high moisture levels cause rapid loss of spore viability.

For any biocontrol programme to be successful, production of sufficient quantities of a good quality inoculum is essential. The production system of entomopathogens depends on the scale of production. For instance, for laboratory studies, relatively small quantities of the inoculum and field-testing during the development of mycoinsecticide could be done at laboratory scale with very sophisticated equipment as it involves standardization of production system. On the contrary, for bulk production of the biocontrol agent, large-scale production involving commercially viable protocols may be needed to cater to the needs of the stakeholders. For the mass production of *B. bassiana*, *P. fumosoroseus* and *V. lecanii*, Sahayaraj and Karthick (2008) evaluated various agricultural produce like rice, wheat, maize, sorghum, finger millet and pearl millet at different temperatures, solid media such as carrot, okra, jack seeds, rice husk and sawdust and liquid media like rice washed water, wheat washed water, coconut water and rice cooked water. Similar efforts were made by Agale et al. (2018) and Gopalakrishnan et al. (1999) for the mass production of *P. farinosus*, *B. bassiana* and *M. anisopliae* to identify cheap sources of nutrition for mass multiplication of these EPF and reported that the production supported multiplication and sporulation of fungi. Mass multiplication of entomopathogens such as *Beauveria bassiana* and *Metarhizium anisopliae* can be done using the diphasic liquid–solid fermentation technique developed for the LUBILOSA (Lutte Biologiquecontre les Sauteriaux, www.lubilosa.org) project (Lomer et al. 1997). Whereas the liquid phase provides active growing mycelia and blastospores, the solid phase supports production of dry aerial conidia. The conidia thus produced can be used either directly as natural granules or formulated as a suitable formulation depending on the target insect-pests.

Field studies on the evaluation of emulsifiable concentrate (EC) of *Metarhizium anisopliae* against arecanut white grubs (late second/early third) revealed that 60 days after application, higher concentration of *M. anisopliae* (Novozyme) at 5 × 109 (3 ml/l) and *M. anisopliae* (Tstanes) at 1 × 109 (3 ml/l), and recommended dose *M. anisopliae* (Novozyme) at 5 × 109 (2.5 ml/l) registered 100, 97.78 and 94.44% population reduction in white grubs as compared to *M. anisopliae* dust application. Use of *Metarhizium* is an effective eco-friendly approach especially the use of EC formulations through drip has lot of potential in arecanut and coconut ecosystem (Vinayaka et al. 2018). Sahayaraj and Namachivayam (2011) evaluated the bioefficacy of *B. bassiana*, *P. fumosoroseus* and *V. lecanii* on the insect-pests of groundnut along with the persistence of these fungi wherein it revealed that pest population and their infestation were significantly reduced in entomopathogenic fungal treatments. Similarly, experiments were carried out to determine bioefficacy of an entomopathogenic fungus formulation-Bioterminator (*Metarhizium anisopliae*)s against termites infesting tea. Experiments were performed in both

laboratory and natural conditions. In the laboratory experiment, mortality data were recorded daily up to 7 days after treatment and at monthly interval for field experiments. Under in vitro conditions it was concluded that Bioterminator was found to be effective (43.28–72.94%) up to 7 days after treatment. Under field conditions, Bioterminator was found to be highly effective (85.21, 84.91 and 85.97%, respectively) in controlling termites and maintain their effectiveness to the desired level up to 9 months (Hoque et al. 2016). Hussain et al. (2011) found that the *M. anisopliae* has effectively reduced the population of termites at the time of sugarcane planting and it also reduced sugarcane bud damage and increased germination. Singha et al. (2011) reported that the strains of *Metarhizium anisopliae* and the strains of *Beauveria bassiana* are effective agents against tea termite, *Microtermes obesi* Holmgren. An experiment under natural conditions was conducted to evaluate the bioefficacy of different mycoinsecticide formulations such as crude, wettable powder and oil-based formulation of *Beauveria bassiana*, *Metarhizium anisopliae* and *Verticillium lecanii* in okra against sucking pests (Harischandra and Naik 2009). The use of entomopathogenic fungi particularly, *Beauveria bassiana* and *Metarhizium anisopliae*, has significant role in the management of white grubs of sugarcane in tropical regions of India. Mycoinsecticide, *M. anisopliae*, at the rate of 8×10^9 conidia per ml was found to be effective in reducing the white grub population and yield, and quality parameters recorded were higher in treated plots compared to untreated plots (Thamaraichelvi et al. 2010). Similarly, the application of *M. anisopliae* at 5×10^{13} spores ha^{-1} combined with FYM gave higher cane yield (Pandey 2013; Hadiya et al 2016; Sharma and Anoorag 2017; Visalakshi et al. 2015). Samuels et al. (1990) reported that *M. anisopliae* at 1×10^{13} spores ha^{-1} application recorded higher cane yield.

9.4.2 Registration Requirements for Mycoinsecticides

Any biopesticide formulation reported to contain pesticidal properties needs to be registered with a competent registration authority. The guidelines for registering biopesticide vary from country to country. In general, the authenticity of biopesticide molecule needs to be proved with its physical, chemical, biological, toxicological, storage and stability properties. The manufacturer needs to generate these data from the credible research institutions and laboratories. A few countries accord registration for the data generated by following Good Laboratory Practices.

Registration authorities look for data on technical active ingredients and formulation. For example, azadirachtin is the active ingredient of any neem formulation. When 3 g of azadirachtin a.i. is formulated with suitable solvent/oil, emulsifier, surfactants, etc., the formulation becomes a brand carrying 0.3% azadirachtin. In case of microbial biopesticides like *Trichoderma viride*, the primary culture becomes its active ingredient. When *Metarhizium anisopliae* spores/mycelia with/without broth is formulated with a suitable carrier, it becomes formulations. Dossier data is required for both formulations and technical active ingredients.

9.4.2.1 Biological and Chemical Properties

The biological and chemical properties of the mycoinsecticide must conform to the laid out standards of biological and chemical properties. They include (i) the organism's common and systemic names, strain/accession number, source of origin, host range and mode of action; (ii) product specifications like appearance, physical state, colour, pH, foaming, solubility or suspension capacity, particle size, viscosity/density; (iii) composition of the formulation including the active ingredients, inert materials, exo/endotoxins and contaminants/impurities; and (iv) manufacturing process and its flow chart, quality control methods and the address of the premises where the manufacturing will be taken up.

9.4.2.2 Bio-efficacy Studies

The formulation needs to be evaluated for its bio-efficacy in laboratory, screen/greenhouse and field by a competent study team under university/research institute approved by the regulatory authorities. The field efficacy shall be carried out against the target pest(s) and crop(s) for two seasons and three locations in order to study its efficacy and dose.

9.4.2.3 Toxicity/Ecotoxicity Studies

Toxicity/pathogenicity of the formulation needs to be carried out on an array of animals/birds/fishes/honey bees/earthworms/beneficial organisms including human health exposure. In some cases, the environmental fate of the formulation, residues, re-entry period, pathogenicity to non-target organisms will also be required to submit.

9.4.2.4 Packing, Storage and Stability Studies

Studies on suitable packing material for the formulation as per international/national guidelines, its shelf life for a period of 6–24 months and stability of the formulation are to be carried out and submitted to the registering authorities.

9.4.2.5 Application, Legal, Labels and Leaflets

Labels and leaflets contain information on the product, active ingredients, compositions, dosage/concentration, handling, disposal, first aid, toxicity indicator, manufacturer name, licence and registration number, dates of manufacturing and expiry. Application needs to be submitted to the registration authority with the above-stated information along with an undertaking for the person responsible for legal.

9.4.3 Benefit-cost ratio and Popularity of Mycoinsecticides in India

Bio-insecticides are less popular among farmers/growers as compared to conventional chemical pesticides due to their (i) slow action; (ii) often act against narrow target pests and (iii) mixed benefit: cost ratio. The benefit-cost ratio of select bio-insecticide, viz. *V. lecanii* and *M. anisopliae*, has been reported to be better than a

few conventional insecticides (Manisegaran et al. 2011; Dixit 2015). Bhadani et al. (2017) got excellent management of custard apple mealybug by applying *Beauveria bassiana* (2×10^6 cfu/gm) formulation at 2.0 gm/lit, and the benefit-cost ratio was 7.1:1. Against chilli thrips, *Metarhizium anisopliae* application at 0.25% yielded a benefit-cost ratio of 15.9:1. Sahayaraj and Namachivayam (2011) have reported a high benefit-cost ratio of 5.01:1 for *B.* bassiana (2×10^8 CFU) when administered at 2 g/lit for management of fruit and shoot borer on brinjal. However, a ratio of 1.93:1 could be realized against *Spodoptera litura* on groundnut. However, in other studies, conventional insecticides were reported to be cost-effective than fungi-based insecticides (Singh et al. 2011; Ajay 2013). The following table gives the overall benefit-cost ratio of bio-insecticides as compared to conventional insecticides and untreated control.

For improving the efficacy of the formulations, combinations of insecticides have been permitted for use in insect-pest management. As on today, there are 13 such combinations available in India. On the contrary, majority of biological pesticides are slow in action as compared to conventional pesticides due to their mode of action. Moreover, being living organisms, the availability of congenial environment for their establishment, multiplication and infestation of the host species is critical for the success of given mycoinsecticide. Under these conditions, the bioefficacy could be enhanced if more than one compatible mycoinsecticides are permitted to be formulated together to harness the synergy between them. As these fungi exhibit different modes of action, the target pests could be managed with better efficacy, when they are given in a single formulation. However, to achieve this, there is a need for amending the registration guidelines. Vast scientific studies across the globe reported improved efficacy of the biopesticide formulations if two or more active ingredients are used (Wraight and Ramos 2017; Sayed and Behle 2017; Chandrasekaran et al. 2015; Mantzoukas et al. 2013). For example, the larval control is reported to be better when the combinations using two or more of the following biopesticides: *Beauveria bassiana*, *Metarhizium robertsii*, *Bacillus subtilis*, *Bacillus thuringiensis* var. *israelensis*, *Bacillus thuringiensis tenebrionis*, botanical blends or neem formulations (Shaalan et al. 2005; Kryukov et al. 2009; Kaur et al. 2015; Mantzoukas et al. 2013). However, there are no approved guidelines so far in India and elsewhere for the combination biopesticide formulations. Provisional registration given in select third world countries has also been withdrawn/suspended and, hence, no commercial combination biopesticides permitted/available across the globe as on date.

Researchers and regulatory authorities are agreeing that biopesticides are relatively safe to mankind, animal and environment as compared to conventional pesticides. As the active ingredients of these biopesticides (botanical and microbial) are naturally derived, they shall be safer than conventional pesticides and their combinations. This includes the bio-safety of the formulation. Organized guidelines are available for registering conventional insecticide combinations, insecticide-fungicide combinations, but not for biopesticides. Therefore, it is essential and mandatory for the registration authorities to open up the gate for combination biopesticides. Another important bottleneck is delay in scrutinizing the

applications. Provisional licences are awarded in 1–3 years of scrutiny. However, permanent licences take about 3–8 years after submitting the applications. In India, 19 biopesticides have been registered and 341 firms have been licensed to produce biopesticides. As per the data of Central Insecticide Board & Registration Committee, Faridabad, India, interestingly, so far as many as 783 provisional licenses for biopesticides have been granted. However, only 22 products have been issued permanent licenses. This also highlights the bottlenecks involved in obtaining permanent licenses by the firms.

9.5 Commercial Products Available

Despite above-mentioned difficulties in commercialization in India and elsewhere, some products are available in the market for use by the farmers. Development of various methods of mass production of infective spores of *M. anisopliae* and the successful techniques for mass culture of this fungus has led to the commercial development of this fungus as a mycoinsecticide. The fungal spores can be grown on sterilized rice in plastic bags for small-scale production and in semi-solid fermentation for large scale. The fungus spores can then be formulated as a dust. Spore viability of *M. anisopliae* decreases with the increase of storage temperatures. At lower temperatures its virulence decreases and it is sensitive to extreme temperatures. Sprayable formulations of *M. anisopliae* have been used for the management of sugar cane meadow spittlebug and leaf-miner of coffee and *Tomaspis saccharina*, froghopper. Bioblast is a commercially available formulation of *M. anisopliae* that is used to management of termites like *Reticulitermes* sp. Similarly, Vertalec (for control of glasshouse aphids) and Mycotal (for glasshouse whitefly) are two commercial products available based on *Lecanicillium*. Both mycoinsecticides are based on species of the fungus genus *Lecanicillium*: Vertalec consists of *Lecanicillium longisporum*, while Mycotal is based on *Lecanicillium muscarium*. Fargues et al. (1996) reported that *Lecanicillium muscarium*-based formulation reduced population of *Trialeurodes vaporariorum*, greenhouse whitefly by 76–96% in Mediterranean greenhouse tomato. In the United States, at present no commercial formulation of *Isaria fumosoroseus* is available, whereas in Europe a commercial product is being marketed under the trade name “PreFeRal.”

In India, so far, 84 formulations of *Beauveria bassiana*, 80 formulations of *Lecanicillium (Verticillium) lecanii* and 33 formulations of *Metarhizium anisopliae* have been registered by the Central Insecticides Board & Registration Committee. Top four companies that have maximum registrations are Agri Life, T-Stanes, Varsha Bioscience and Technology India Pvt Ltd and Viswamithra Bio-Agro Pvt Ltd. A list of commercial formulations along with their target insect-pests is provided in Table 9.1. However, the table is not exhaustive. Also, listing of some products does not automatically imply that they are being promoted.

Table 9.1 Main target pests of commercially available mycoinsecticides

Fungus	Product	Formulation	Target pests
Beauveria bassiana	BotaniGard, Boverol, Naturalis-L, Proecol Mycotrol, Beauverin Bio-Power, Myco-Jaal	WP, SC	Lepidoptera (diamondback moth, beet armyworm, cabbage looper, cutworm, etc.), Coleoptera (scarab beetle grubs, weevils, coffee berry borer, cutworms, etc.), Heteroptera (psyllids, stinkbug, plant bugs, leafhoppers, mealybugs, aphids, whitefly, etc.), Thysanoptera (western flower thrips)
Beauveria brongniartii	Betel, Schweizer Beauveria	WP	Lepidoptera (diamondback moth, beet armyworm, cabbage looper, cutworm, etc.)
Lecanicillium lecanii	Mycotal, Bio-Catch Vertalec, Phule Bugicide	WP	Heteroptera (stinkbug, aphids, whitefly, plant and leafhoppers, mealybugs), Thysanoptera (western flower thrips, onion thrips), Orthoptera
Lecanicillium longisporum	Vertalec		Homoptera, Hemiptera, Thysanoptera
Metarhizium anisopliae	Bio-Magic, Green muscle, Biocane, Metarril, Ory-X, Bioterminator	WD	Coleoptera (scarab beetle grubs, weevils), Blattodea (termites), Heteroptera (leafhoppers), Orthoptera (grasshoppers), Lepidoptera (cutworms)
Metarhizium flavoviride var. *flavoviride*	BioGreen, BioCane	WP, G	Coleoptera (scarab beetles, weevils), Orthoptera (grasshoppers and locusts), Blattodea (termites)
Isaria fumosorosea	Preferal, Priority, Futureco, Nofly, Ph-97	WP, WDG	Heteroptera (whiteflies, aphids, etc.), Lepidoptera (tomato moth), Acari (rust mites, spider mite, etc.), Lepidoptera
Hirsutella thompsonii	ABTEC Hirsutella	Liquid	Coleoptera, Diptera, Hemiptera, Acari
Nomuraea rileyi	–	WP	Larval stages of lepidopteran pests, especially Spodoptera.

9.6 Limitations for Use of Mycoinsecticides

Over the years, farmers have started using biopesticides in general and EPF in particular for management of insect-pests. Similarly, continuous efforts were underway to isolate potential isolates of EPF for efficient pest control. These efforts coupled with increasing concern for the immediate environment have resulted in registration of commercial products world over including India. However, there are some inherent and extraneous limitations that are hindering the spread of these products among farmers. Some of the bottlenecks are listed below:

- Since these are biological systems, the effect of application is dependent on several factors such as application dosage, time and site.

- After application, there is a time lag based on the mode of action of the EPF and hence, the result could not be seen immediately as in case of pesticides.
- There is a need for continuous research as the isolates display variability and hence, many isolates need to be evaluated for identification of potential candidate isolates.
- Quality of the product, stability of the formulation and its consistent field efficacy are some of the issues often encountered in promotion of these products.
- Pesticides are pushed more aggressively compared to these products.
- Some of the biopesticide formulations are laced with chemical pesticides leading to loss of faith on purity of these products.
- The inordinate delay in registration and inadequacy in regulatory are issues of concern especially in many developing countries

9.7 Conclusions and Future Perspectives

Although biocontrol method of managing the insect-pests was first discovered several decades ago, biocontrol strategies for the effective control of insect-pests have come to limelight with the increasing concern for the environment and sustainability issues. Thus, today, several commercial products are in vogue for the management of insect-pests. Further, increasing demand for organic agricultural products has also increased demand for use of these biocontrol agents. Entomopathogenic fungi are in a way nature's gift to mankind as they are keeping the insect-pest populations under control by infesting them.

Brazil, the United States, China, Mexico, Australia and France are the important consumers of biopesticides. The consumer awareness is relatively better in these countries. Africa and Southeast Asia are the emerging biopesticide markets. Many farmers in the developing countries are ignorant about efficacy of biopesticides against insect-pests. Further, most of the products currently available in the market are based on limited isolates and hence, often fail to perform in diverse ecosystems. However, due to the penetration of effective formulations over time, there has been a gradual increase in use of these products. Further, promotion of organic agriculture is also opening up the markets for biopesticides. The way forward thus should focus on (i) exploiting the vast biodiversity of the fungi so that new and efficient isolates are formulated, (ii) developing cheaper mass multiplication systems and stability of the product, (iii) formulations for stability under different agroecological conditions, (iv) innovative approaches to improve shelf-life by using novel molecules/materials, (v) promoting stakeholder participatory approach in developing the product so that the stakeholder is aware of the products right through and thus building faith in the product, (vi) identification of abiotic stress tolerant strains to overcome climate change issues, (vii) liberalization of registration procedures to encourage more products to penetrate the market, (viii) more scientific approach to registration through biocontrol laboratories and technocrats join hands together, (ix) recognition of more laboratories for generation of data required for registration of biopesticides, (x) establishment of data banks on biopesticides for public access and

(xi) establishment of region-wise subdivisional offices for scrutiny of the registration applications and promote doing ease of business. Among the 171 products, the most common products are based on *Beauveria bassiana* (33.9%), *Metarhizium anisopliae* (33.9%), *Isaria fumosorosea* (5.8%) and *B. brongniartii* (4.1%). At present about 75% of all listed products are registered, or in the process of registration or commercially available, whereas 15% are not available in the market.

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Microbial Diversity of the Sundarbans, the World's Largest Tidal Mangrove Forest, and Its Bioprospects

10

Kaushik Biswas and Joydeep Mukherjee

Abstract

Sundarbans, the world's largest tidal mangrove forest, lies in the delta of the Ganges, Brahmaputra, and Meghna rivers. The ecosystem is dynamic and the biodiversity is enormously rich. The debris and the waste materials generated by local industry as well as domestic sources flowing through the rivers accumulate in this deltaic region. This detritus-based hugely productive ecosystem supplies large amounts of organic substances to the resident organisms and builds up a productive ecosystem. Mangroves are one of the striking sources of microbial diversity. Microbes have been explored as a potential source of bioactive compounds for novel pharmaceutical applications. Till date, very little work has been carried out on the microbial diversity of the Sundarbans. Few attempts have been made to explore the culturable and un-culturable microorganisms of this ecosystem. Till date, two novel species, *Streptomyces sundarbansensis* sp. nov. and *Streptomyces euryhalinus* sp. nov., have been reported from this region. Several other actinomycetes were also isolated and a few bioactive compounds have been purified. Moreover, industrially important enzymes such as protease, esterase, and ribonucleases have been purified and characterized from bacteria isolated from the Sundarbans. Many halophilic cyanobacterial strains have been isolated; among them, *Oxynema aestuarii* sp. nov. was reported as a new cyanobacterial species. Further, the diversity of un-culturable bacteria and archaea of the Sundarbans have been explored by applying the metagenomic approach, and the sequence data have been analyzed using bioinformatics tools. Several studies have documented *Proteobacteria* as the dominant phylum, while *Firmicutes*, *Chloroflexi*, *Planctomycetes*, and *Actinobacteria* were found seasonally at different locations of the mangrove forest. Looking into the dynamic microbial

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community composition, the Sundarbans ecosystem has a great potential for the discovery of novel microbial species and deliver bioactive compounds for industrial, medical, and environmental applications.

Keywords
Sundarbans · Microbial diversity · Fungi · Bacteria · Archaea · Cyanobacteria · Bioactive compounds

10.1 Introduction

In the world map, the Sundarbans covers the territories of Bangladesh and India (Fig. 10.1), having an area of nearly 10,200 km^2 (Gopal and Chauhan, 2006). It is one of the richest biodiversity hotspots in the world. Sundarbans, situated on the delta of the rivers Ganges, Brahmaputra, and Meghna on the Bay of Bengal, is the world's largest tidal halophytic mangrove forest. The name "Sundarbans" has been derived from the "Sundri" tree which means beautiful. The Indian part of the Sundarbans was recognized as world heritage site by the International Union for Conservation of Nature (IUCN) in 1987, and Bangladesh part of the Sundarbans was also declared as a world heritage site in 1997. Part of the Sundarbans situated in Bangladesh was included in the Ramsar list of wetlands of international importance in 1992. Many rivers like Hooghly, Matla, Hariabhanga, Raimangal, and Saptamukhi flow through the Indian Sundarbans. The mixing of freshwater with saline water followed by the segregation of sediment is an important characteristic of this estuary. In addition, constant changes in geomorphological and topographical properties of the substratum play an important role in its diversity. Being an estuarine site, the Sundarbans delta provides constant supply of nutrients to the biogeochemical cycles leading to enormous production of biological resources (Meire et al. 2005). Seasonal variations and spatial differences in biogeochemical process also influence the ecosystem significantly. These phenomena help the Sundarbans to produce and maintain its rich biodiversity.

The mangroves include approximately 60–75% of the world's subtropical and tropical coastlines (Holguin et al. 2001). Mangrove communities or trees are present in the tropical or subtropical region as transitional coastal ecosystem, which are highly productive and dynamic (Chakraborty et al. 2015). The term mangroves indicates both communities and trees. The communities are comprised of bacteria, microalgae, fungi, invertebrates, birds, and mammals. This immensely rich biodiversity and productive ecosystems supply large amounts of organic matter to the contiguous coastal water and animal kingdom (Holguin et al. 2001). Waste material is one of the main sources of nutrients, and it creates a comprehensive food cycle for the organisms. In nature, waste material comes from fallen leaves and wood of mangroves which are degraded by microbes (Alongi 1989; Moran and Hodson, 1989). Mangroves are thought to be the global hotspots of microbial diversity due to their variable features like salinity, temperature, light, tidal flooding, high rainfall, and

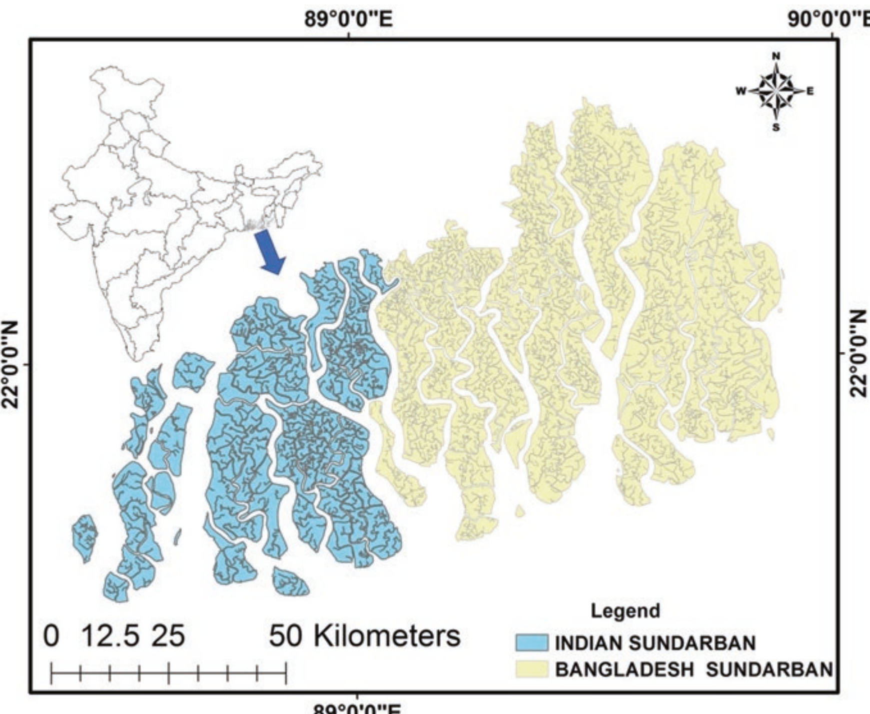

Fig. 10.1 Map showing the Indian and Bangladesh Sundarbans

other physicochemical factors (Andreote et al. 2012; Feller et al. 2010). Mangroves are also a conservatory of many marine and terrestrial organisms. Microbes are the main players for the transformation of nutrients and organic matter in the mangrove forest (Holguin et al. 2001). It is very obvious that microbes, nutrients, and plants have a bona fide relationship for the conservation of the mangrove ecosystem. Bacteria are the important players for the chemical cycling of carbon, nitrogen, sulfur, and phosphorus in the mangrove forest (Toledo et al. 1995; Vazquez et al. 2000; Rojas et al. 2001). Mangroves harbor many microorganisms and plants which could be the source of various novel bioactive compounds due to their specific environmental and ecological features (Xu et al. 2014). Many novel bacteria and fungi have been identified and explored from different mangrove forests (Biswas et al. 2017; Wei et al. 2011). Mangrove forests have been reported to be a source of several novel bioactive compounds including antibiotics (Xu et al. 2014; Arumugam et al. 2010; Huang et al. 2008).

Sundarbans mangrove is well known for its diversity of zooplankton, phytoplankton, invertebrates, and mammals. Endangered Royal Bengal tiger (*Panthera tigris*) is a special feature of its ecodiversity. Indian Sundarbans is a habitat of 364 types of fishes, 497 types of insects, 11 types of amphibians, 47 types of mammals, and 270 types of algae (Danda et al. 2017). Microbes play a leading role in

maintaining the highly productive mangrove forest ecosystem. Microbes take part directly in nutrient transformation, nitrogen fixation, solubilization of phosphates, reduction of sulfates and nitrates, and in the generation of organic matter (Ghosh et al. 2007; Santos et al. 2011). The microbes from mangroves are already established as novel and prolific source of antibiotics, enzymes, and other bioactive compounds. From the Sundarbans mangrove forest, several new species of bacteria, fungi, and virus have been discovered. Sundarbans detritus ecosystem and climate are nurturing the growth of various genera and species of fungi. Sundarbans mangrove harbors the second largest group of fungi among all marine fungi (Sridhar 2004). Exploration of microbial diversity of Sundarbans and research on drug discovery would contribute to better human health and in solving ecological or environmental problems.

10.2 Diversity of Culturable Microbes and Their Role in Habitat/Environment

10.2.1 Bacterial Diversity

Bacteria play an important role in maintaining biodiversity and ecosystems. The Sundarbans is a detritus-enriched ecosystem which is partly produced and maintained by bacteria. Microbial diversity of mangrove ecosystems has not been studied systematically, and the Sundarbans is not an exception to this. Little information is available on the microbial diversity of the Sundarbans (Ghosh et al. 2010). Very few investigations have been carried out till date on the diversity of culturable and non-culturable bacteria and their bioactive compounds. Bacterial diversity of the Indian Sundarbans from the perspective of biotechnological applications was first attempted by Dr. Joydeep Mukherjee's group in Jadavpur University, Kolkata (India) and Prof. Maitree Bhattacharyya's group in the University of Calcutta, Kolkata (India). Few more groups have recently initiated work on the microbial diversity of the Sundarbans and its potential biotechnological applications. It is not possible to get the real picture of bacterial community present in a specific ecosystem with the help of culture-based traditional techniques alone. 16S rRNA-based techniques are now being employed in order to overcome problems encountered using the traditional cultivation-based methods. 16S rRNA sequencing-based method is not the gold standard technique for assessing bacterial diversity due to its inadequate resolution capacity. According to Zeyaullah et al. (2009), only 0.1–1.0 % of microorganisms can be cultured from any environment. In order to know microbial diversity, modern metagenomic approach along with next-generation sequencing must be employed. Andreote et al. (2012) have reported applicability of such techniques in assessing microbial diversity of Brazilian mangrove forest.

Biswas et al. (1986) reported isolation of a number of cellulolytic, pectinolytic, amylolytic, lipolytic, and pectinolytic bacteria from the Sundarbans. In the same year, Bhowmik et al. (1986) described isolation of 48 bacterial strains from decomposable litter and different animals. Choudhury and Kumar (1996) reported

isolation of ten different microorganisms from coastal region of the Sundarbans; among them, three were enteropathogens. The enteropathogens *Vibrio cholerae* non-01(CT^+), *Pseudomonas aeruginosa*, and *Escherichia coli* (ETEC) could grow in the presence of nickel, cobalt, copper, cadmium, silver, lead, and zinc. These organisms were found to be resistant to 5–10 µg/ml antibiotics and adapted to the extreme conditions like high salinity and toxic metals. Choudhury and Kumar (1998) isolated *Klebsiella pneumoniae* from the alimentary canal and gills of a shrimp *Penaeus monodon* which was collected from the Haroa region of the Sundarbans. Ten isolates were able to tolerate 10 mM silver, nickel, cobalt, cadmium, lead, copper, and zinc and were resistant to antibiotics like erythromycin, ampicillin, furazolidone, and penicillin. Bhattacharya et al. (2000) isolated strains of *Vibrio parahaemolyticus* from the shrimp species *Penaeus monodon*. Eight strains of *Vibrio parahaemolyticus* displayed resistance to erythromycin, penicillin, streptomycin, and sensitivity to nalidixic acid, nitrofurantoin, tetracycline, and norfloxacin. Among these, five strains were resistant to ampicillin and kanamycin, while six were resistant to cephalexin. All the eight strains of *Vibrio parahaemolyticus* showed resistance to 10 mM cadmium, zinc, and lead. Roy et al. (2002) isolated petroleum-degrading bacteria; 0.08–2.0% of culturable heterotrophic bacteria, viz., *Pseudomonas*, *Mycobacterium*, *Klebsiella*, *Acinetobacter*, *Micrococcus*, and *Nocardia*, were capable of degrading crude petroleum hydrocarbons. Among them, five strains from *Pseudomonas*, *Mycobacterium*, and *Nocardia* degraded 47–78% of crude oil. One strain of *Pseudomonas* BBW1 showed highest (75%) oil-degrading capacity in 72 hours. This strain showed plasmid-mediated resistance to a few antibiotics and metals. Saha et al. (2005) isolated an actinomycete MS 3/20 from Lothian Island of the Sundarbans, which was later identified by Biswas et al. (2017) as *Streptomyces euryhalinus* sp.nov. It is a gram-positive, aerobic, non-motile, salt-tolerant actinomycete, and spores are spiny, arranged in a retinaculum-apertum manner, and produce yellowish brown to red aerial hyphae. Saha et al. (2005) purified a potent antimicrobial compound of molecular weight 577.49 from the strain MS 3/20 and proposed molecular formula as $C_{20}H_{35}NO_{18}$. This compound showed significant antimicrobial activity against a wide range of gram-positive and gram-negative bacteria, yeast, and molds (Saha et al. 2005). Saha et al. (2006) isolated another actinomycete from the same niche, strain MS 1/7, which produced a bioactive compound having molecular weight 300.2 and molecular formula of $C_{20}H_{28}O_2$ (Fig. 10.2a). Later, Arumugam et al. (2011) identified the strain MS 1/7 as *Streptomyces sundarbansensis* which was the first validly published bacterium from the Sundarbans. A smoke-flavored compound 2-allyloxyphenol of molecular weight 150 ($C_9H_{10}O_2$) [Fig. 10.2b] was purified and characterized first time as a natural product although it is known as a synthetic compound and chemical intermediate (Arumugam et al. 2010). This bioactive compound showed antimicrobial activity against bacteria and fungi. Interestingly, Djinni et al. (2013) identified *Streptomyces sundarbansensis* from marine brown alga *Fucus* sp. from Algerian coastline and reported an anti-MRSA novel polyketide. One more strain MS 310, isolated from the Sajnekhali Island of Sundarbans, showed 99% 16S rRNA gene sequence similarity with *Streptomyces paravallus* which is known as a gram-positive

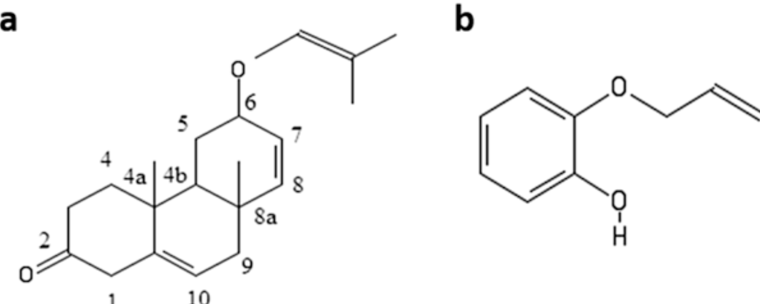

Fig. 10.2 Proposed structure of the active compounds from actinobacterium MS1/7 isolated from the Indian Sundarbans. (**a**). 4a,8a-Dimethyl-6-(2-methyl-propenyloxy)-3,4,4a,4b,5,6,8a,9-octahydro-1H phenanthren-2-one (Saha et al. 2006) (**b**) 2 allyoxyphenol (Arumugam et al. 2010). (Reprinted with the permission of Springer Nature under license number 4399260962874 and 4399260291039, respectively)

spore-forming actinomycete that produces actinomycin D. Strain MS310 was cultivated in a rotating disk bioreactor (RDBR) at one revolution per day and produced actinomycin D without elaborating any other analogues (Sarkar et al. 2008). The use of RDBR for the production of antibiotics from the marine organisms originated from the concept of "niche-mimic bioreactor" principle of providing the same environment for the bacteria from where it was isolated. Such cultivations enhance the production of antimicrobial compounds when compared to cultivations in standard bioreactors. The onset of antibiotic production by strain MS310 was 20 h in RDBR as compared to that of 55 h in a standard stirrer tank bioreactor (STBR) as reported by Sousa et al. (2002).

Another investigation on the diversity of actinomycetes for studying antimicrobial activities was that carried out by Sengupta et al. (2015) in three different locations of the Indian Sundarbans [Gadkhali (station A), Bonnie camp (station B) and Kalash (station C)]. Fifty-four actinomycete isolates were screened for antimicrobial activities; 2 isolates from 25 isolated from Gadkhali, 1 isolate among 6 from Kalash, and 6 among 23 isolates from Bonnie camp displayed antimicrobial properties. Gadkhali is subjected to high anthropogenic influence, while Bonnie camp is pristine and relatively undisturbed region. 16S rRNA sequence analyses of these strains exhibited highest similarity with *Streptomyces* genus. One strain designated as SMS_10 revealed 92.5% similarity with *Corynebacterium auris* DSM 328^{T} and 93.57% with *Streptomyces albogriseolus* NRRLB-1305^{T}in the EzTaxon database. EzTaxon (https://www.ezbiocloud.net/) is an online database applied for the identification of prokaryotes based on 16S rRNA gene sequence. The strains showed different types of sporophore ornamentation with a dissimilar pattern of carbon source utilization. According to the study, two isolates SMS_SU13 and SMS_SU21 were grouped as moderate halophiles, while strain SMS_7 was a borderline halophile, with 99% 16S rRNA sequence similarity with *Streptomyces tendae* that produces nikkomycin (Brillinger, 1979). These three strains displayed potent antibacterial and antifungal activities. The strain SMS_SU21 showed highest

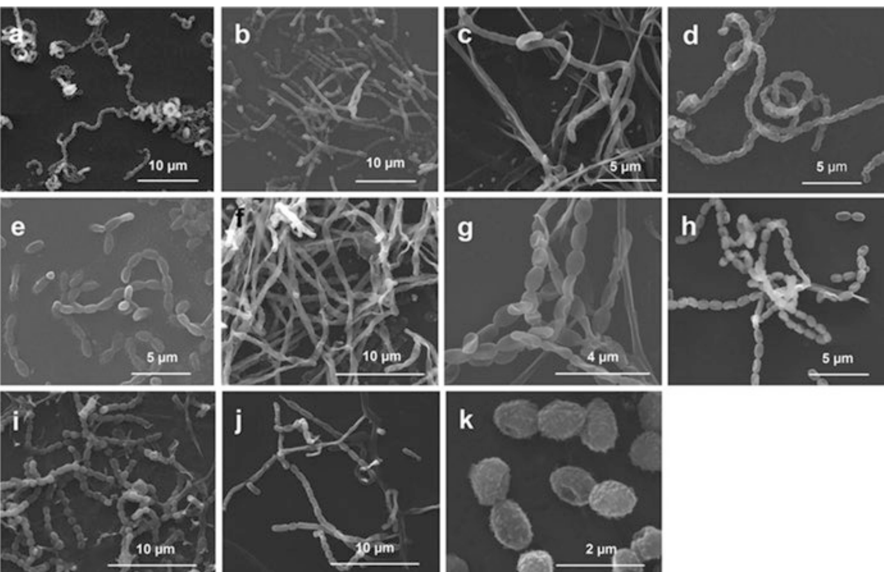

Fig. 10.3 Scanning electron micrographs showing spore arrangement of eleven Streptomycetes isolated from the Indian Sundarbans (**a**) H531, (**b**) AH251, (**c**) H524, (**d**) AS151, (**e**) ASH253, (**f**) ASH151, (**g**) H562, (**h**) SH521, (**i**) ASH853, (**j**) H532, and (**k**) ASH252 (Mitra et al. 2011). (Reprinted with permission of Springer Nature license number 4399260781530)

antimicrobial (MIC 0.05 mg/ml) and antioxidant activities of IC_{50} value 0.242 ± 0.33 mg/ml. GC-MS analysis of crude extract of strain SMS_SU21 showed the presence of 2, 2-propyl-*N*-ethylpiperidine; 4-dichloromethyl-5 6-epoxy-2-methoxy-4-methyl-2-cyclohexenone; 1,3-cyclopentanedione compounds, which could be attributed to its antimicrobial and antifungal property. Further characterization of the bioactive compounds from this strain may provide new antimicrobials. Mitra et al. (2008), for the first time, established the relationship between distribution of actinomycetes and antagonistic potential of the samples collected from near-sea region, intertidal region, and mangrove forests of the Indian Sundarbans. Intertidal regions having alluvial soil contained highest number of actinomycetes, while sandy sediments yielded lowest numbers. Authors concluded that antagonistic potential of site depended on the tides. Mitra et al. (2011), in another study, reported the isolation of eleven actinomycetes (Fig. 10.3) from the nine high antagonistic potential sites of the Sundarbans. Eleven strains showed antimicrobial activity against gram-positive and gram-negative bacteria and different fungi.

Sana et al. (2006) isolated a true marine bacterium isolate DGII from the sediment samples of Lothian Island of Sundarbans, which was categorized on the basis of 16S rRNA gene sequence as a Gamma-proteobacterium. This is a gram-negative, rod-shaped bacterium which grew in the presence of 30% sodium chloride, but optimum growth was recorded at 2.0% sodium chloride. Authors purified and characterized a serine protease enzyme from isolate DGII which could be useful in the detergent industry. The enzyme showed activity over a broad range of pH (6.0–11.0)

and temperature (30–70 °C). The enzyme displayed stability in commercial detergents, laboratory detergents, wide range of solvents, bleaches, and oxidizing and reducing agents. The activity of the enzyme was enhanced in presence of Ba^{2+} and Ca^{2+} and inactivated by Co^{2+}, Zn^{2+}, and Hg^{2+}. Another strain was isolated from the Lothian Island of the Sundarbans designated as BSE01 and identified by 16S rRNA analysis as a *Bacillus* sp. (Sana et al. 2007). The strain grew in MB2216 medium containing up to 10% sodium chloride and produced esterase in the same medium containing 1% (w/v) sodium chloride. BSE01 was an aerobic, gram-positive, halotolerant, rod-shaped bacterium of the family *Bacillaceae*. An esterase enzyme was purified from the strain which was tolerant to 30–70% dimethyl sulfoxide and exhibited activity on p-nitrophenyl acetate, ethyl acetate and alpha isomer of naphthyl acetate. The enzyme was inactivated in presence of Ca^{2+}, Fe^{2+}, Hg^{2+}, reducing agents, and detergents. The enzyme could be useful in industry and bioprocesses. Sana et al. (2008) isolated a *Bizionia* sp. (BSR01) from the Lothian Island of Sundarbans belonging to *Cytophaga-Flavobacterium-Bacteroides* phylum. The strain grew on MB2216 medium containing 13% of sodium chloride and was a gram-negative, aerobic, halotolerant marine bacterium. A uracil-specific RNase enzyme was purified from the strain BSR01 which may find potential application as a reagent in a molecular biology laboratory. Ramanathan et al. (2008) studied microbial diversity of three locations of the Sundarbans, namely, Canning, Jharkhali, and Pakhiraloy, and found highest microbial presence at Canning (29.83×10^6 CFU/g) in comparison to the other two locations, Jharkhali (1.11×10^6 CFU/g) and Pakhiraloy (4.71×10^6 CFU/g). Canning also showed the presence of highest phosphate-soluble bacteria (14.08×10^4 CFU/g), free-living nitrogen-fixing and nitrate-forming bacteria (13.67×10^4 CFU/g and 50.12×10^4 CFU/g) than the other two locations. Phosphate-solubilizing bacteria contribute to the pool of phosphate ions from the sediment or water to plants, and nitrogen-fixing bacteria are well known for increasing the amount of nitrogen to the plants. Interestingly, Pakhiraloy showed the highest number of cellulose-degrading bacteria (45.15×10^4 CFU/g) which indicates that an environment sustained with high organic litter increased the redox potential of the ecosystem that ultimately led to the decomposition of organic matter. By using culture-dependent method, Das et al. (2012) reported the presence of cellulose-decomposing bacteria, sulfate-reducing bacteria, phosphate-solubilizing bacteria, nitrogen-fixing bacteria, fungi, and nitrifying bacteria from three different locations of the Sundarbans. The three locations were deep forest region Patharpratima (location 1), rooted region Lothian Island (location 2), and unrooted region Saptamukhi estuarine mouth (location 3). Cellulose-decomposing bacteria were found to be the dominant group in the deep forest region (location 1), which may be due to high organic carbon content of the region. Post-monsoon period showed the presence of highest number (6.189×10^6 CFU/g) of cellulose-degrading bacteria compared to the monsoon (5.491×10^6 CFU/g) and pre-monsoon periods (4.756×10^6 CFU/g). The sulfate-reducing bacterial load was found to be maximum (0.570×10^6 CFU/g) in pre-monsoon and minimum in monsoon (0.249×10^6 CFU/g) at deep forest region (location 1) than other two regions. The maximum (0.634×10^6 CFU/g) free-living nitrogen-fixing bacteria were found during

pre-monsoon and minimum (0.452×10^6 CFU/g) during monsoon as compared to the other two stations. In the year 2011–2012, Basak et al. (2015a, b) conducted a detailed study on the microbial diversity at the surface and subsurface layers of sediment of three different mangrove locations, namely, Jharkhali (location A), Sahidnagar (location B), and Godkhali (location C), of the Sundarbans with seasonal variations. Microbial community composition was correlated with the seasonal changes. The study revealed dominance of *Proteobacteria* phylum in the samples at all three locations which was recorded at about 57–72.4% in December 2011 and 49–79 % in July 2012. Dominance of *Proteobacteria* in Sundarbans mangrove forest and other mangrove regions was previously reported (Ghosh et al. 2010, Dos Santos et al. 2011). Surface samples contained high numbers of *Proteobacteria* in monsoon, while subsurface samples showed higher number in post-monsoon in all the three study regions. Among *Proteobacteria*, dominant classes were *Deltaproteobacteria* and *Gammaproteobacteria*. The presence of *Deltaproteobacteria* clearly indicated the anaerobic nature of the sediment which supported the selection of sulfate-reducing organisms of the same class. Basak et al. (2015a) described the abundance of aerobic bacteria belonging to the order *Myxococcales* in the surface samples from Sahidnagar (location B) and suggested that eco-restoration influenced the mangrove vegetation and supply of oxygen (Clark et al. 1998). Other two stations showed the presence of sulfur-reducing anaerobic bacteria such as *Desulfobacterales*, *Desulfuromonadales*, and *Bdellovibrionales*. This may be attributed to the anthropogenic factors affecting mangrove vegetation of locations A and C. The most prevalent groups were represented by the orders *Chromatiales*, *Legionellales*, and *Xanthomonadales* of the phylum *Proteobacteria* under the class *Gammaproteobacteria.* Among these orders, anaerobic *Chromatiales*, known as purple sulfur bacteria and an indicator of anthropogenic intervention, are prevalent in the Sundarbans. The presence of *Legionellales* in the sediments is an index of human interference in the region. Among *Alphaproteobacteria*, the most dominant members represented the orders *Rhodospirillales*, *Rhodobacterales*, *Rhizobiales*, and *Sphingomonadales* in all three stations. In this order, *Rhizobiales* is a symbiotic, nitrogen-fixing bacteria, while *Sphingomonadales* are aromatic hydrocarbon-degrading bacteria (Liang and Lloyd-Jones 2010), and *Rhodobacterales* is a primary surface colonizer found in a temperate coastal marine sediment and marine water (Dang et al. 2008). Basak et al. (2015a) also reported higher abundance of *Actinobacteria* phylum (1.9–23.2% in July 2012) in the subsurface samples in monsoon session and residence of *Planctomycetes*, *Acidobacteria*, *Chloroflexi*, *Cyanobacteria*, *Nitrospira*, and *Firmicutes* from all the samples collected from three locations in the two seasons. The population of *Acidobacteria* was found distributed evenly throughout all the sampling locations in both the seasons due to the neutral pH of Sundarbans sediment. Various groups of diazotrophic bacteria were isolated from the saline soils of different locations of mangrove forest and agricultural field of the Sundarbans under Indian territory; and the effect of these bacteria on the crop production has been studied (Barua et al. 2012). Among 40 isolates, 4 were selected for nitrogen fixation, indole acetic acid production, and phosphate solubilization efficiency. With the help

of 16S rRNA sequence analysis, strain SUND_ BDU1 was characterized in the genus *Agrobacterium* and the strains SUND LM2, Can4, and Can6 were grouped in the genus *Bacillus*. Bioinoculants were prepared using farmyard manure along with four bacterial strains, and significant increase in yield of crop production was observed. On the basis of field trial on rice and Lady's finger, strain Can6 demonstrated the highest yield and productivity compared to the other strains. Other three strains also increased the yield and productivity of the crops significantly. Salt-tolerant bioinoculants may be substituted for the chemical fertilizers in the saline coastal area for the sustainable agriculture (Barua et al. 2012).

Recently, work on the reduction of Cr (VI) contamination in rice plants growing in pots using a Cr(VI)-resistant *Staphylococcus* isolated from Sundarbans paddy field has identified *Staphylococcus sciuri* as the key biotransforming bacterium (Dutta et al. 2017). The chromium-contaminated soils treated with this bacterium removed 71 ± 3% Cr (VI) and 65 ± 2% of total Cr after 8 weeks. In bacterium-treated soils, total Cr removal from rice seeds of plants was observed to be 78 ± 4% when compared with control rice seeds. The bacterium brought out 95 ± 5% Cr (VI) reduction in rice seeds cultivated in bacterium-treated soil when compared with control rice seeds. This study could help in bioremediation of chromium in the cultivated soils of the Sundarbans for sustainable agriculture. Soil samples were collected from mangrove tree rhizosphere at Bonnie Camp (21°4953.581″N 88°36″44.860″E) of the Indian Sundarbans for the screening of arsenic-resistant bacteria (Mallick et al. 2018). Two strains AB402 and AB403 among 25 isolates were selected for the identification and bioremediation of As(III) contamination. Strains AB402 and AB403 were identified using 16S rRNA sequencing analysis as *Kocuria flava* and *Bacillus vietnamensis*. Strains showed maximum resistance to As (III), As (V), and Cu (II). Arsenic adsorption of the strains was confirmed through the energy-dispersive X-ray (EDX) of scanning electron microscopy where a distinct peak was observed and transmission electron microscopy exhibited the accumulation of intracellular As by recording dark electron-dense region. Determination of exopolysaccharide (EPS) content showed 27.5 and 315 μg/ml of protein which could be attributed to As adsorption property of both strains. Both strains were also observed for their biofilm formation ability. However, when strains were treated with As, biofilm ability was reduced but they formed biofilm significantly in the presence of 2 mM and 50 mMAs(III) and As(V). Importantly, both the strains promoted the growth of rice seedlings and reduced the arsenic accumulation in the plants. A bacterial strain SuMS_N03 isolated from the saline environment of Sundarbans (21.69N, 88.565 E) was identified as *Staphylococcus warneri* (Nag et al. 2018). The intracellular protein extract of this bacterium was used to prepare gold nanoparticles (GNP) of 15–25 nm size at room temperature. This synthesized GNP in the presence of excess $NaBH_4$ catalyzed the complete reduction of 2-nitroaniline, 4-nitroaniline, 2-nitrophenol, and 4-nitrophenol and exhibited no significant by-products. It could be useful for the degradation of pollutants as a green renewable catalyst and for the treatment of wastewater. Srinivas et al. (2013) isolated a strain $AK13^T$ from a sediment sample of mangrove soil in Namkhana (21°45′39.35″ N 88° 13′48.05″ E) and identified as *Silanimonas mangrovi* sp. nov.

under the class Gamma-proteobacteria (Lee et al. 2005). The bacterium was a gram-negative, aerobic, motile, and rod-shaped bacterium which grew in 8% sodium chloride, and the G+C content was 55.2 mol%. Vector-borne disease like dengue and malaria have become threats to the tropical countries. From the different localities of Indian Sundarbans, soil samples were collected and screened for the mosquitocidal activity against larvae of *Anopheles sundaicus*, *Aedes aegypti*, and *Culex quinquefasciatus* (Chatterjee et al. 2018). Bacterial strain SB1 was identified as *Bacillus thuringiensis* which showed effectiveness against *A. sundaicus* larvae (late 3rd instar) having LC_{50} and LC_{90} as 5.73×10^8 CFU/ml and 4.78×10^9 CFU/ml, respectively. Similar values against *A. aegypti* were LC_{50} 5.82×10^8 CFU/ml and LC_{90} 7.58×10^9 CF/ml, while those against *Culex quinquefasciatus* larvae were found to be LC_{50} 6.32×10^8 CFU/ml and LC_{90} 9.8×10^9 CFU/ml.

Very few studies were carried out to explore the bacterial diversity of Bangladesh part of Sundarbans, and a few reports are available. Arifuzzaman et al. (2010) reported screening of actinomycetes from Karanjal region of the Bangladesh Sundarbans. A total of 55 isolates of actinomycetes were obtained from the screening and categorized into four genera: *Actinomyces*, *Nocardia*, *Micromonospora*, and *Streptomyces*. Among these isolates, 20 showed antimicrobial activity against *Shigella boydii*, *Shigella flexneri*-AN-31153, *Shigella sonnei*, *Pseudomonas*, *Shigella dysenteriae* type-1, *Vibrio cholerae*-0139, *Salmonella typhi*-Ao-12014, *Plesiomonas*, *Hafnia* sp., *Vibrio cholerae*-OGET, and *Escherichia coli* 186LT. Screening of pigment-producing bacteria of the soil samples from three locations of Bangladesh, namely, Tambulbunia, Herbal Nadir Tek, and Katka, was conducted (Saha et al. 2017). Katka showed the highest bacterial count of 3.38×10^7 CFU/g and the lowest count of 2.68×10^7 CFU/g were observed at Tambulbunia. It was also noticed that pH 6.5, temperature 37 °C, and salt concentration of 2–14% were ideal conditions for the bacterial growth and pigment production. Bacteria represented by the yellow (30%) and orange (30%) were dominant pigment colors among 30 isolates studied for the pigment production. Among 30 strains, 7 isolates were classified into the genus *Bacillus*, and 1 isolate showed similarity with *Marinococcus halophilus*. Another study was conducted in the Sundarbans of Bangladesh to screen the antibacterial producing marine bacteria and on the basis of colony morphology they were placed in the genus *Streptomyces*. These isolates were grouped into five color series on the basis of color of aerial mycelia, substrate mycelia, and soluble pigment, where orange series (30.7%) and gray series (28.2%) were dominant (Sarker et al. 2015). Among 39 isolates, 24 showed broad-spectrum antimicrobial activity against 3 gram-positive and 4 gram-negative bacteria. Brown series (70%) showed highest antibacterial activities followed by 63.6% of the gray series, 50.0% of white series, 58.3% orange series, and 50.0% of red series. Yet, another study by Sarker et al. (2015) described isolation of bacterial strains from the Bangladesh Sundarbans, and on the basis of antibacterial activity, one strain ANAM-5 was selected for the further study. This strain showed 99.64% similarity with *Planctomyces brasiliensis*; however, morphological and physicochemical studies showed differences when compared to *Planctomyces brasiliensis*. Polyphasic taxonomic characterization for conclusive identification of the strain may be needed.

10.2.2 Cyanobacterial Diversity

Cyanobacteria are well known for their role in nitrogen fixation. Diversity and abundance of cyanobacteria, availability of biomass, and their role in nitrogen fixation and denitrification were investigated by Essien et al. (2008) and Lee and Joye (2006). Isolation of halophilic cyanobacteria of the Indian Sundarbans and their antimicrobial property was first studied by Pramanik et al. (2011). Authors conducted the study on 14 locations of Sagar Island (21°44′ 7″ N,88°7′2″E) in November 2006 and 12 locations on Lothian Island (21°39′ 1″N, 88°19′37″E) of the Indian Sundarbans. Eight halophilic cyanobacteria were selected from the total 60 isolates on the basis of antimicrobial activity. The eight isolates were designated as AP3, AP9F, AP17, AP20, AP24, AP25, AP3b, and AP9U (Fig. 10.4). Among these, six cyanobacteria were grouped into *Lyngbya-Phormidium-Plectonema* (LPP), and the other two were classified into *Oscillatoria* and *Synechocystis* genera, on the basis of 16S rRNA gene sequence data. All of these strains grew at low salinity as well as at 3.2–8.2% salinity but antimicrobial property was observed at 3.2% salinity. The extracts of these strains demonstrated MIC of 0.25 to 0.50 mg/mL against *S. aureus*, *E.coli*, *B. subtilis*, and *P. aeruginosa*, whereas the MIC values were found in the range 0.50–1.0 mg/mL against clinical isolates.

Out of eight strains isolated from the Sundarbans (Pramanik et al. 2011), two strains AP17 and AP24 were characterized and identified up to species level using a polyphasic approach. 16S rRNA analysis showed 99% similarity with the one species of the newly formed *Oxynema* genus (Chatchawan et al. 2012). Based on morphological and ultrastructural characterization and analysis of the secondary structures of 16S-23S ITS regions, the strains were assigned as the second novel species under the genus *Oxynema*, namely, *Oxynema aestuarii* (Chakraborty et al.

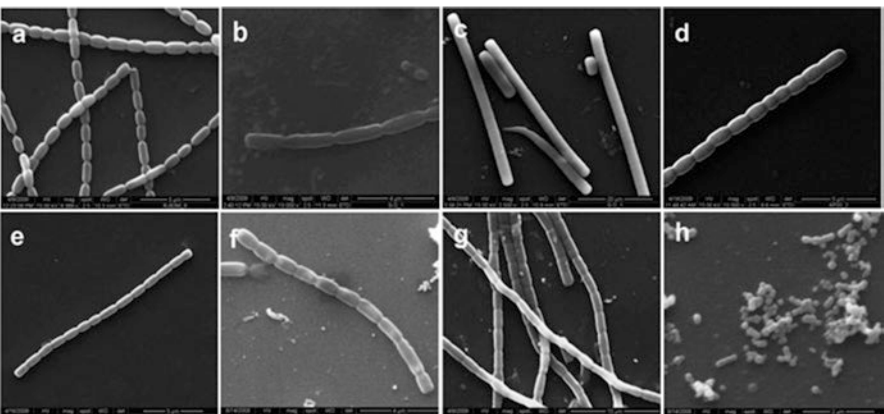

Fig. 10.4 Scanning electron micrographs of eight halophytic cyanobacterial isolates from the Indian Sundarbans (**a**) AP3, (**b**) AP9F, (**c**) AP17, (**d**) AP20, (**e**) AP24, (**f**) AP25, (**g**) AP3b, and (**h**) AP9U (Pramanik et al. 2011). (Reprinted with permission of John Wiley and Sons, licence number 4399261316556)

2018, article in press). This cyanobacterium was characterized morphologically as long, straight, unbranched, filamentous (cell length 1.5–2.5 cm, cell width 2.03–2.45) with apical cells narrowed, pointed, and slightly curved without any calypta. Ultrastructural observation displayed radial arrangement of thylakoids. The ITS region of *O. aestuarii* was constituted of D1-D1′ helix (62 nucleotides), D2 region with a spacer (46 nucleotides), V2 spacer (12 nucleotides), Box B helix (55 nucleotides), Box A (12 nucleotides), D4 region (7 nucleotides), and V3 helix (51 nucleotides). Veerabadhran et al. (2018) studied growth, biofilm formation, chlorophyll a formation, EPS production, and antimicrobial activity of cyanobacterial strains isolated from the Sundarbans by Pramanik et al. (2011), namely, AP17 (*Oscillatoria* sp.), AP3b (*Leptolyngbya* sp.), and AP3U (*Chroococcus* sp.) in a patented conico-cylindrical flask (CCF) made of polymethylmethacrylate (PMMA) and standard Erlenmeyer flask (EF). Biofilm formation in CCF recorded (average dry weights) was highest in strain AP17 (*Oscillatoria* sp.) [0.0665 ± 0.02 g], and there was no biofilm formation in the EF. Strains AP17 and AP3U showed a high chlorophyll a accumulation in biofilms (1378 ± 211 and 183.18 ± 5.7 μg/g) as compared to planktonic growth (657 ± 140 and 65.72 ± 1.9 μg/g), although AP3b showed higher chlorophyll a in planktonic form (849.7 ± 2.8 μg/g) than in biofilm (548.3 ± 3.8 μg/g). AP3b extract displayed higher antimicrobial activity against *B. subtilis* than that of AP3U in CCF configuration, whereas AP17 extract did not exhibit antimicrobial activity. TLC profile of biomass extracts of AP17 and in CCF and EF was dissimilar. LC-MS spectra of these cyanobacteria revealed numerous biological activities like antimicrobial antifouling, enzyme inhibition, antioxidant, anti-inflammatory, anti-diabetic, immunomodulatory, anticancerous, antiquorum sensing, and others. Debnath et al. (2017) studied the cyanobacterial diversity of the Indian Sundarbans and characterized 12 genera of 22 morphospecies belonging to the Trical non-heterocystous members like *Geitlerinema*, *Coleofasciculus*, and *Leptolyngbya*. Among these, one morphotype *Leptolyngbya indica* sp. nov. isolated from arsenic rich soil of a rice field was reported as a novel species.

10.2.3 Fungal Diversity

Fungi are diverse group of organisms which play crucial roles in ecology and bioprocesses like nutrient recycling, litter decomposition, soil formation, and bioremediation (Gadd, 2007). Fungi have potential to become important candidates in industrial, medicinal, and biotechnological applications as it could be a source of novel bioactive compounds (Albersheim 1966; Bennett 1998; Acharya 2007; Chatterjee et al. 2011; Khatua et al. 2013; Bhadury et al. 2006). According to an estimate, there are 5.1 million of fungal species on Earth (O'Brien et al. 2005), and mangroves are known to be natural habitat of fungi (Jones and Alias 1996). Raghukumar (1973) first reported marine fungi isolated from Indian mangroves, *Dactylospora haliotrepha* (Ascomycota). Rai and Chowdhury (1978) isolated 184 fungal species from mangrove swamps and suggested organic matter to be mainly responsible for the fungal activity in preference to salinity and anaerobic conditions.

Rhizosphere, the region under the influence of the plant root, is one of the favorable niches of mangrove fungi. Garg (1981) described host-specific fungi, whose distribution and frequency were dependent on the plant. Chowdhury et al. (1982) reported that rhizosphere soils were responsible and supportive of fungal growth. This study concluded that *Aspergillus* was the most dominant genus, and the other dominant genera were *Fusarium* and *Penicillium*.

Sengupta and Chaudhuri (1990, 1994, 2002) studied the association of arbuscular mycorrhizal (AM) fungi with mangrove and associated plants of estuarine habitat. AM colonization was observed by the authors in four species of pioneer salt marsh plants (Sengupta and Chaudhuri 1990). In another study, Sengupta and Chaudhuri (2002) observed the presence of root endophytic fungi in 54 different species of plant in four successive stages of plant growth. Among these, 31 species were mangrove and mangrove associates which showed the presence of root endophytic fungi, and 23 were non-mangrove species which showed presence of AM endophyte. Riverine sediment brought the AM fungal isolates in the mangrove ecosystem as spores or root fragments which eventually adapted into the saline nature of the region. Authors isolated seven different Glomalean spore types from the rhizosphere soil mangrove and associated plants. These were identified as belonging to *Glomus mosseae* (Giovannetti et al. 1993), *G. fasciculatum*, *G. macrocarpum* (Songachan and Kayang 2013), *G. multicaulis*, *Gigaspora margarita*, and *Acaulospora mellea*, and one isolate was unidentified. Among these five, *G. mosseae*, *G. fasciculatum*, and *Gigaspora margarita* were reported as the dominant species. Kumar and Ghose (2008) studied host-plant relationship for the effects on spore density and frequency on mycorrhizal colonization in the roots. Authors studied 15 mangroves and selected 27 sites of 6 different regions of the Sundarbans. The study concluded that host-plant relationships were directly responsible for spore density and frequency of mycorrhizal colonization in the root, and it was not affected by tidal inundation of the area. Increase in the availability of soil phosphorus decreased the mycorrhizal frequency and spore density. Authors (Kumar and Ghose 2008) further reported the dominance of genus *Glomus* in different regions of the Sundarbans. Leaf-inhabiting fungi of mangrove plants of the Indian Sundarbans were surveyed by Pal and Purkayastha (1992a) for the first time and seven fungi were reported. In another study, Pal and Purkayastha (1992b) reported isolation of two new species. Furthermore, Purkayastha and Pal (1998), with the help of light microscopy and scanning electron microscopy, observed the uredinial stage of *Skierka agallocha*, the fungus on leaves of *Excoecaria agallocha.*

Three strains of *Pestalotiopsis versicolor* were isolated from *Ceriops decandra* by Bera and Purkayastha (1992) from different regions of the Indian Sundarbans. The effects of pH on growth, salt tolerance, and nature of sporulation were observed. Increase of salt concentration in the medium generally caused decrease of cellulolytic activity of fungi (Garg, 1982). The highest enzyme activity was observed in *Chaetomium globosum* and *Aspergillus terreus* at 0 and 6% salt levels, respectively, while the lowest cellulolytic activity was demonstrated by *Aspergillus niveus* and *Scopulariopsis brevicaulis* at 0 and 6% salt level. De et al. (1999) isolated 16 fungi from the plants of the Sundarbans mangrove forest and studied their growth pattern

in the presence of tannin. At a concentration of 0.2% tannin, growth of all fungi (*P. agallochae*, *C. senegalensis*, and *E. psidii*) was inhibited, whereas at 0.05 %, tannin stimulated the growth of the three fungi. Two fungi showed maximum 99% reduction in viscosity of buffered pectin substrate and one fungus showed minimum 13% reduction. Dutta et al. (2013) carried out a macrofungal diversity study in the Indian Sundarbans (21°32′–22°40′N, 88°85′–89°E) and recorded the presence of total of 62 species across 27 families and 46 genera. The frequently present species in the region were *Auricularia auricula*, *Ganoderma lucidum*, *Tricholoma crassum*, *Schizophyllum commune*, and *Dacryopinax spathularia*. *Ganoderma* and *Tricholoma* exhibited greatest species richness in the region. Among all the macrofungi, 55% were saprophytic. Dutta et al. (2011) reported isolation of *Leucocoprinus birnbaumii* from Kumirmari (22.19° N; 88.93° E) of the Sundarbans. Rajasekar et al. (2012) reported isolation of *Fusarium* and *Aspergillus* species from the Sundarbans. Authors tested the antimicrobial activity of the isolated fungi against different bacterial species and maximum inhibition against *E. coli*, *Pseudomonas* sp., and *Vibrio* sp. as observed. Nandy et al. (2014) isolated few fungi from the Jharkhali of the Indian Sundarbans which were morphologically identified as *Aspergillus niger* and *Penicillium* sp.; both were tested for the biodegradation of fishery industry waste. Removal of hexavalent chromium from the aqueous solution was demonstrated by *Aspergillus* sp. isolated from the Chakraborty and Sengupta et al. (2018). Removal of chromium (VI) was standardized by optimizing different parameters like inoculum size, temperature, pH, etc. The highest removal of chromium (VI) was achieved using potato dextrose broth at 27 °C and pH 4.0. Fungal diversity of Sundarbans has not been studied extensively and ample opportunity exists for the discovery of novel species.

10.3 Diversity of Non-culturable Microbes and Their Role in Habitat/Environment

10.3.1 Bacterial Diversity

According to Zeyaullah et al. (2009), only 0.1–10.0% microorganisms present in nature can be cultured. It is quite impossible to provide specific conditions for the microbes to grow in cultivation-based methods due to several limitations. All bacteria present in any environmental sample, therefore, cannot be studied by the culture-dependent method. New advancements in metagenomics with sequencing technology and bioinformatics analysis allow to study non-culturable bacteria and facilitate better understanding of bacterial diversity in any environment (Tringe et al. 2005; Lauber et al. 2009; Edwards et al. 2006). These techniques have been applied in Brazil and China to know the microbial diversity of mangrove forests (Andreote et al. 2012; Jiang et al. 2013).

To know the diversity of bacterial community in Sundarbans mangrove forest, Ghosh et al. (2010) applied the culture-independent approach. Sediment samples were collected from Netidhopani (21°55′13″ N, 88°44′46″ E) of the Indian

Sundarbans. Eight main phyla of bacteria were recorded by applying the metagenomic approach. Among eight phyla, *Proteobacteria* was the major phylum that included alpha, beta, gamma, and delta classes. In sequencing analysis, 29 sequence types (comprised 58%) in D16S_pMOS library and 57 sequence types (comprised 71%) in DUni_pMOS library were detected as *Proteobacteria*. *Gammaproteobacteria* was the most abundant, and it accounted for 59% in D16S_pMOS and 77% DUni_pMOS libraries. Many *Gammaproteobacteria* clones showed the sequence similarity with hydrocarbon and oil-degrading bacterial communities. From D16S_pMOS and DUni_pMOS libraries, 14 and 5% proteobacterial sequence types were *Betaproteobacteria*, respectively. Among these, a few displayed sequence similarity with *Betaproteobacteria* which had been isolated previously from oil-contaminated locations. In these two libraries (D16S_pMOS and DUni_pMOS), six *Alphaproteobacteria* were found. Sequence-type clone D16S_119 showed 95% sequence similarity with an *Alphaproteobacteria* clone MPCa6_A10 isolated from the sponge *Microciona prolifera* from the Chesapeake Bay. In D16S_pMOS and DUni_pMOS libraries, 2.1% and 7% of clones showed similarity with deltaproteobacterial sequence, respectively. Many of the deltaproteobacterial sequences showed similarity with other strains or clones having sulfate reduction property. Six clones containing 2% of the D16S_pMOS library showed sequence similarity with *Cytophaga-Flexibacteria-Bacteroides*. One clone D16S_176 showed sequence similarity with the *Flexibacteraceae*, recently isolated from Venice Lagoon anoxic sediments. Only two clones showed sequence similarity with *Chloroflexi* in D16S_pMOS. This clone showed sequence similarity with uncultured *Chloroflexi* which was recently isolated from the mangrove sediment of Xiamen, China. Six clones in D16S_pMOS library and three clones in DUni_pMOS library demonstrated sequence similarity with *Planctomycetes*. Only six clones grouped into *Actinobacteria*, which accounted for 2.5% in the DUni_pMOS library; two showed sequence similarity with uncultured actinobacterial clones. The observations of this study corroborated well with previous reports from culture-based method for studying microbial biodiversity (Chakraborty et al. 2015; Basak et al. 2015a, b) although sampling site was different, but within the Sundarbans. Another metagenomics-based study was carried out by Basak et al. (2015a, b) to know the microbial diversity of the Indian Sundarbans. Sampling was done from a depth of 9 m at Bonnie Camp (21° 49′ 53.581″ N, 88° 36′ 44.860″ E) and 5 m at Dhanchi (21° 42′ 06.41″ N, 88° 25′ 54.682″ E), respectively. V1–V3 region of the 16S rRNA gene was sequenced for assessment of the biodiversity. In both locations, *Proteobacteria* and *Firmicutes* were the most abundant bacterial communities. This study indicated the presence of 67% and 19% of *Proteobacteria* at Bonnie Camp at depths of 9 m and 5 m. In case of Dhanchi, *Proteobacteria* were 36% at 5 m depth. Bonnie Camp sample contained 76% and 5% *Firmicutes* at depths of 5 m and 9 m, respectively, whereas Dhanchi at a depth of 5 m showed presence of 27% *Firmicutes*. The other major phylum detected at Dhanchi was *Chloroflexi* (11%). Likewise, Bonnie Camp samples included 6% and 3% *Chloroflexi* population at 9 and 5 m depths, respectively (Fig. 10.5). Basak et al. (2016) carried out a

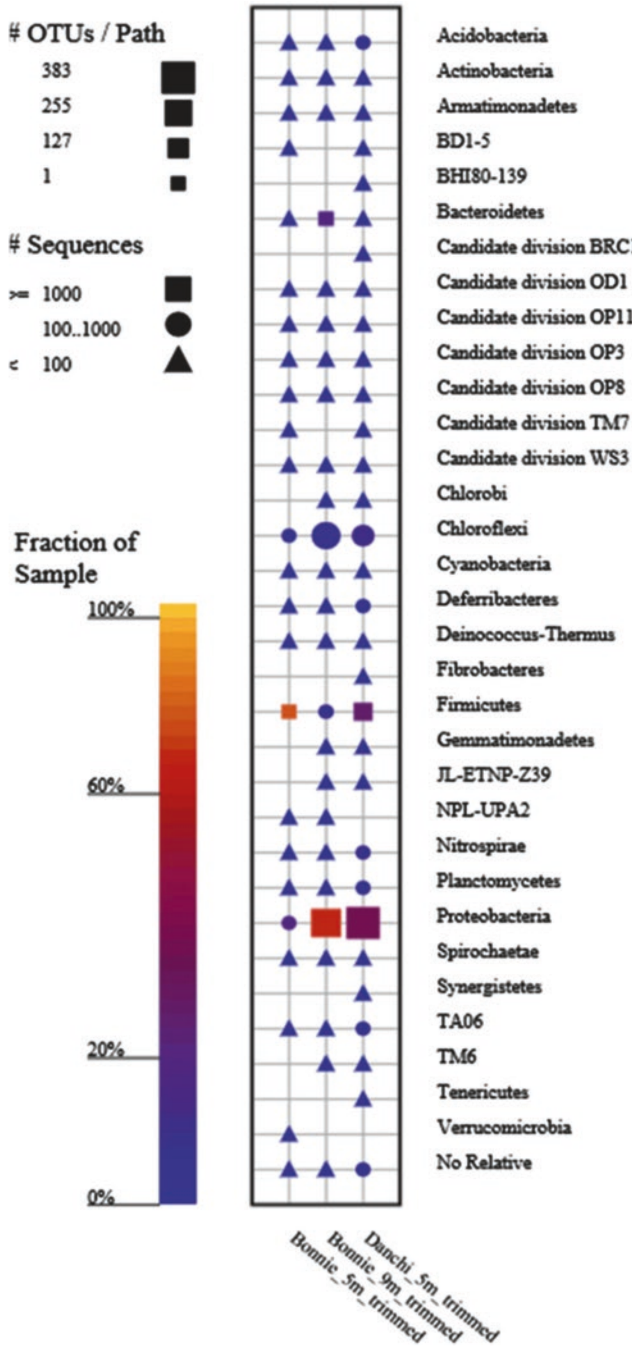

Fig. 10.5 Presence of bacterial communities in soil sediment samples of different locations in the Indian Sundarbans (Basak et al. 2015a, b). (Reprinted with permission of Elsevier, license number 4399270489904)

metagenomic study for exploring the bacterial biodiversity at Dhulibhashani (21°37′40.837″N, 88°33′ 47.762″E) of the Sundarbans in monsoon and pre-monsoon seasons. Forty-two different phyla in monsoon and 39 different phyla in pre-monsoon were recorded. Study reported *Proteobacteria* as a major phylum as described in other locations by the same research group. The report claimed the presence of 53% *Proteobacteria* in pre-monsoon at 2 cm and 31% at 32 cm, while the similar values in monsoon were 44% at 2 cm and 38% at 32 cm depths. Study revealed *Bacteroidetes* as the second major phylum representing 19% (2 cm depth) and 2% (32 cm depth) in monsoon and 15 (2 cm depth) and 5% (32 cm depth) bacterial population in post-monsoon, respectively. *Firmicutes*, *Actinobacteria*, and *Planctomycetes* were also present in Dhulibhashani in both seasons. Very recently, Ghosh and Bhadury (2018) published the diversity of bacterial community at two stations in the Sagar Island (named as Stn.1: 21° 40′ 44.4″ N, 88° 08′ 49.5″ E and Stn2: 21° 40′ 40.6″ N, 88° 09′ 19.2″ E) by culture-independent approach. This study again confirmed the dominance of *Proteobacteria* in the region as a major phylum represented by the classes *Alphaproteobacteria*, *Betaproteobacteria*, *Gammaproteobacteria*, and *Deltaproteobacteria*. *Bacteroidetes* and *Firmicutes* constituted the next major phyla in both the stations as well as in monsoon and post-monsoon seasons. Additionally, *Desulfovibrio*-like sequence was found in both the stations and in monsoon and post-monsoon seasons. In general, sulfate-reducing bacteria like *Desulfovibrio* were observed in coastal ecosystem, and they play an important role in the oxidization of carbon sources like petroleum hydrocarbons (Nealson 1997; Stahl et al. 2002).

10.3.2 Archaeal Diversity

Archaea thrive in the extreme environments and form the third domain of life that plays a significant role in maintenance of biogeochemical cycles because they are the major players of ammonia oxidation in the environment (Jarrell et al. 2011). Knowledge of the diversity of Archaea in mangroves is limited (Yan et al. 2006). To the best our knowledge, diversity of Archaea in Sundarbans was studied by Bhattacharyya et al. (2015). Authors analyzed the diversity of Archaea of Sundarbans following the metagenomic approach by collecting samples from Godkhali (station A, 22°06′32.570″N 88°46′22.220″E), Bonnie camp (station B, 21°49′53.581″N88°36′44.860″E), and Dhulibhashani (station C; 21°37′40.837″N 88°33′47.762″E). Authors assigned the 16 S rRNA sequences into two archaeal phyla and five archaeal classes. Euryarchaeota were found to be the most dominant phylum (36–60%), and Thermoplasmata was the most abundant class in all samples (39–62%). Thaumarchaeota was reported as the second most abundant phylum in all the samples. Halobacteria class, within euryarchaeal sequences, was represented by the genera *Halosarcina*, *Halorientalis*, *Halolamina*, *Halorhabdus*, *Halogranum*, *Haloferax*, *Halomarina*, *Halorussus*, *Haloplanus*, and *Halarchaeum*. These observations corroborated with the presence of polyaromatic hydrocarbons in the sampling locations as halobacteria are well known for their hydrocarbon-degrading

characteristics. The class Methanomicrobia representing the genera *Methanosarcina*, *Methermicoccus*, *Methanocella*, *Methanococcoides*, *Methanosalsum*, *Methanolobus*, and *Methanogenium* were recovered. Authors did not find the presence of any member from archaeal phyla such as Crenarchaeota, Korarchaeota, Nanoarchaeota, and Nanohaloarchaeota. Halobacteria displayed high abundance in the subsurface layer samples from Godkhali and Bonnie camp, while in Dhulibhashani, Methanomicrobia was dominant in the subsurface sediment. Dhulibhashani was predominantly inhabited by Methanosarcinaceae and Methanobacteriaceae in surface and subsurface regions. Methanomicrobiata were responsible for the sulfate reduction and methanogenesis in the mangroves.

10.4 Potential Applications

Multiple drug resistance (MDR) is now a serious concern for the health system worldwide. Resistant microorganisms may cause death of ten million people in the year 2050 (de Kraker et al. 2016). Therefore, discovery of new antibiotics or secondary metabolites is very necessary. Marine microorganisms could be an alternative source, and marine-derived bioactive compounds are more effective against resistant bacteria (Donia and Hamann 2003; Marwick et al. 1999). Unexplored regions with unique ecology could be the new resources for novel microorganisms. Mangrove forests are relatively unexplored for their bacterial diversity and discovery of new metabolites. It has already been reported that various compounds of potential medicinal or other applications can be isolated from mangrove actinobacteria and fungi (Xu et al. 2014; Arumugam et al. 2010; Wu et al. 2018).

Saha et al. (2005) purified a potent antimicrobial compound of molecular weight 577.49 from strain MS 3/20 and proposed as 2,3-dihydroxy-4-(2,3,5-trihydroxypentanoylamino)-butyric acid 3-[3-(2,3-dihydroxy-propoxycarbonyl)-2,3-dihydroxy-propoxycarbonyl]-2,3-dihydroxy-propyl ester (molecular formula $C_{20}H_{35}NO_{18}$). This compound showed significant antimicrobial activity against a wide range of gram-positive and gram-negative bacteria, yeast, molds, and multidrug-resistant bacteria with MIC in the range of 0.45–0.50 μg/ml. At concentration ten times more than MIC, it lysed only 1.4% of human erythrocytes, which is an important index of its safety and use, though toxicity in animals need to be studied. This compound should be characterized chemically in more detail and its antimicrobial activity has to be determined n vivo. Saha et al. (2006) purified another bioactive compound from the strain MS1/7 of molecular weight 300.2 and proposed the name 4a,8a-dimethyl-6- (2-methyl-propenyloxy)-3,4,4a,4b,5,6,8a,9-octahydro-1H phenanthren-2-one. Later, the strain was identified by the Arumugam et al. (2011) as *Streptomyces sundarbansensis* sp. nov., the first validly published actinomycete from the Sundarbans mangrove forest. The compound showed broad-spectrum antimicrobial activity against MDR bacteria as well as other bacteria and fungi with MIC value of 3.5–4.0 μg/ml. Furthermore, 54% inhibition of growth of human leukemia cell line (HL-60) at 0.05 μg/ml was observed. A smoke-flavored compound 2-allyloxyphenol of molecular weight 150 (molecular formula $C_9H_{10}O_2$) was

purified and characterized for the first time as a natural product by Arumugam et al. (2010), which was previously known as a synthetic compound and chemical intermediate. This bioactive compound showed antimicrobial property against bacteria and fungi. Importantly, this compound had strong antioxidant property of IC_{50} value 22 ± 0.12, though higher than eugenol 7.5 ± 0.0.15. Authors proposed the application of 2-allyloxyphenol as a food preservative and in oral mouthwash preparations. An anti-MRSA new polyketide compound [2-hydroxy-5-[(6-hydroxy-4-oxo-4Hpyran-2-yl) methyl-2-propylchroman-4-one] was isolated from the same actinobacterial strain found in the Algerian coastline by Djinni et al. (2013). This polyketide compound could be useful in the treatment of infectious disease though further studies are required. Actinomycin D, an already well-known antibiotic, was also recovered from the strain MS310 of Sundarbans (Sarkar et al. 2008). This study advocated the "niche-mimic bioreactor" system for the production of antibiotics from bacteria. Authors successfully demonstrated early onset of production of Actinomycin D when MS310 was cultivated in the RDBR compared to cultivation in the standard stirred tank bioreactor (STBR). Authors attributed the higher surface area per unit reactor volume in the RDBR in comparison to the STBR for the attachment of bacteria and biofilm formation, which actually promoted enhancement of the antibiotic production.

Sana et al. (2006) isolated a Gamma-proteobacterium which produces a serine protease enzyme which is capable of removing the recalcitrant blood and egg strains completely from cotton fabric in dry and wet wash operation. It could be used in food processing industry for hydrolysis of soy protein and gelatin and in leather industry for dehairing processes. Further, an esterase enzyme was purified by Sana et al. (2007) from *Bacillus* sp. The enzyme can be useful in industry because of its thermostability; tolerance to solvents particularly its high stability in DMSO can make a potential candidate as immobilized biocatalyst in nonaqueous bioprocesses. Sana et al. (2008) isolated another enzyme from *Bizionia* sp. isolated from Sundarbans. A uracil-specific RNase enzyme was purified from the strain BSR01 which could be used as a source of DNase-free RNase. Chatterjee et al. (2018) isolated *Bacillus thuringiensis*, which could be useful for its mosquito larvicidal potential to prevent the vector-borne diseases as a substitute/supplement to chemical insecticides. Research of Dutta et al. (2017) provided an important opportunity toward bioremediation of chromium from rice. In the same tone, Mallick et al. (2018) provided direction of arsenic remediation using bacteria. Nag et al. (2018) described an insightful solution for bioremediation and wastewater treatment by applying nanobiotechnology. Authors synthesized a stable GNP using intracellular protein from the estuarine bacterium *Staphylococcus warneri.* Authors claimed a recyclable nanocatalyst which effectively degraded nitro-aromatic toxic pollutants. Further research efforts are needed for future applications.

10.5 Conclusions and Future Perspectives

Till date, a few bacteria, fungi, Archaea, and cyanobacteria have been isolated from the Sundarbans region. So it is essential to isolate and identify the microorganisms because characterization of microorganisms will help us to know their nature and metabolite profiles. Mangrove-derived microorganisms produce several bioactive molecules for a variety of applications (Arumugam et al. 2010; Debbab et al. 2011; Huang et al. 2012). Many bacteria have been isolated from the region particularly *Streptomyces* species (Saha et al. 2005, 2006; Mitra et al. 2011), but their identification to the acceptable level and isolation of bioactive compounds had not been done. Mitra et al. (2011) reported many strains, but their identification and isolation of bioactive compounds are incomplete, and it would be worthwhile to carry forward the research on these strains. It is well known that marine *Streptomyces* produce novel bioactive molecules of different pharmacological applications. After Sana et al. (2006, 2007, 2008), no reports on enzymes of bacteria isolated from Sundarbans have been published. Therefore, there are enormous opportunities to undertake research in this area. Fungal biodiversity has been studied by a few groups, but efforts have not been made for investigating fungal bioactive molecules, although there are several reports on bioactive or antimicrobial compounds from mangrove-derived fungi elsewhere (Abdel-Lateff et al. 2003; Dhulipala et al. 2004).

A very few detailed investigations have been carried out on microbial diversity of the Sundarbans and its biotechnological applications till date. On the contrary, extensive research efforts have been made on mangrove microbes in Brazil and China that led to the discovery of several new microbes and novel metabolites. Comparatively, a few new bacteria, cyanobacteria, fungi, and archaea have been described from the Sundarbans. Culturable bacterial diversity was explored in a few locations of the Sundarbans. The limited metagenomic studies suggested non-culturable bacterial diversity in the Sundarbans. Many locations of the Sundarbans remain unexplored. A very few bioactive molecules and enzymes have been reported from the microbes isolated from the Sundarbans. The pressing demand is to explore microbial diversity for novel bioactive molecules. The problem of antibiotics resistance could be overcome by exploration of microbial community of the Sundarbans mangrove forest for novel and effective antibiotics. The biodiversity and ecosystem of Sundarbans are facing several threats. Population density, human intervention, global warming, climate change, sea-level rise, and increased salinity are the threats to the conservation of biodiversity and ecosystem of the Sundarbans. The pressing need of the time is to conserve the biodiversity, ecosystem, and integrity of the Sundarbans.

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Rhizospheric Microbiome Engineering as a Sustainable Tool in Agriculture: Approaches and Challenges

11

Shubham Dubey and Shilpi Sharma

Abstract

Rhizosphere is considered to be supportive of a high microbial diversity because of rich exudation from plant roots. Roots recruit a specific microbial community in this zone, which is beneficial to the plant. Potential of plant growth promoting rhizobacteria (PGPR) has been harnessed since years to serve as bioinoculants. Despite being an eco-friendly alternative to chemicals, and hence of crucial importance in sustainable agriculture, the amendment with single or multiple strains of PGPR has its own set of limitations. After decades of single (and multiple) strain amendments in agriculture, the newer approach is to engineer plant and/or microbiome in the rhizosphere to ultimately lead to enhanced growth of the plant and its ability to alleviate stress. With the recent concept of "holobiont" these two components are no longer separate entities. The present chapter focuses on engineering the rhizospheric microbiome by two approaches, viz. synthetic microbial communities and plant-mediated selection, so as to favour the respective plant's growth. Studies that have attempted the approach have been critically presented. Also, the current limitations in taking the approaches to field have been discussed.

Keywords

Synthetic microbial communities · Rhizosphere · Plant-mediated selection · Microbial diversity

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11.1 Rhizosphere

In the current perspective, plant is no longer viewed as an independent unit. It harbours microorganisms in the phyllosphere, as well as rhizosphere, latter is the region around a plant root that is directly influenced by root exudates so as to recruit a rich microbiome and is considered to be a highly dynamic system. It can be sub-divided into three zones, viz. rhizoplane (surface of root), endorhizosphere (extending from rhizoplane inside the root), and ectorhizosphere (extending from rhizoplane outside into the soil). It is a sight of innumerable interactions amongst microbial members, and between plant and microbe. Rhizosphere releases various compounds (rhizodeposits) into its neighbouring environment that in turn attract a larger proportion of plant growth-promoting rhizobacteria (PGPR), which help the plant to grow (Bais et al. 2006; van Der Heijden et al. 2008). The outcome of an array of interrelationships between microorganisms, and that they share with the plant, is eventually responsible for the overall fitness of the host. The term "holobiont" has been coined for the host (in this case the plant) and its microbiome.

11.2 Rhizospheric Microbiome

Rhizosphere is a home to vast number of microorganisms including plant beneficial, plant pathogenic, as well as human pathogenic organisms. The major reasons responsible for the accumulation of pathogenic strains in the rhizosphere in its congenial environment, e.g. higher carbon content, and protection from desiccation (Tyler and Triplett 2008). The diversity of microbes is higher in the rhizosphere (10^8–10^9 bacteria g^{-1} of soil) as compared to the bulk soil. The microbes are very closely associated and adapted with the plant root and so are referred to as the plant's other genome (Mendes et al. 2013). Due to the lack of culturing techniques the exact composition of rhizosphere has not been elucidated, with only 5–10% of the microbes in the rhizosphere being as-yet culturable. According to available data, most of the studies have reported Proteobacteria to be the most dominant group in rhizospheres, followed by Firmicutes. In fact the latter has been observed to dominate in habitats like Antarctic (Teixeira et al. 2010). Actinobacteria, Acidobacteria and Bacteroidetes follow the other two dominant phyla.

Diverse group of microorganisms serve the purpose of plant growth promotion by various mechanisms like increasing nutrient availability, forming a barrier against phytopathogens and a protectant against biotic and abiotic stresses (Quiza et al. 2015). The microbes present in the rhizosphere play a crucial role in the evolution of plant, and also contribute to the functions of the host plant (Fitzpatrick et al. 2018). Mycorrhiza and rhizobia, which are found in the rhizosphere, help plants with the uptake of phosphorus and nitrogen, respectively. Besides, the microbiome aids the weathering of minerals and the degradation of residual matter. Some microbial members help the host plant by protecting it against pathogens. There are several mechanisms by which this is achieved, e.g. competition for nutrients and synthesis of antibiotics and lytic enzymes which kill the pathogens (Doornbos et al.

2012). Rhizospheric microbiome aid the plant roots in acquisition of trace essential elements, such as iron by the production of siderophores (Hider and Kong 2010). It also triggers the plant defense mechanism by activating the plant immune response by the process of induced systemic resistance (ISR) (van der Ent et al. 2009). Additionally they have a role to play in helping the plant to survive under different types of abiotic stresses.

11.3 Limitations of Currently Practiced Approach of Amendment with Bioinoculants

It has been since decades that the potential of PGPR has been harnessed as amendments in the form of bioinoculants. Bioinoculants have proven to be eco-friendly alternative to chemicals with the latter exerting a long lasting negative impact on environmental health. This sustainable approach has been adopted to serve plant growth promotion through both direct (e.g. nutrient sequestration) and indirect (e.g. non-target impact on resident microbiome, biocontrol) mechanisms. Despite proven benefits, the technology has not picked up in expected fashion. Farmers have lost confidence in the same, and hence they still rely on chemical fertilizers and pesticides to enhance plant growth and productivity.

There are certain lacunae in this approach, and that stands upright with mono-inoculation, or even with a consortium constructed with a group of two or more bioinoculants. Direct introduction of a bioinoculant into rhizospheric zone is expected to confront a considerable measure of competition for its survival in another natural surroundings (new habitat), and have more noteworthy odds of being decreased or diminished in the new habitat. Introducing bioinoculants may cause disturbances to the already existing equilibrium in the rhizosphere, thereby disrupting beneficial natural interactions (plant-microbe and microbe-microbe interactions) present in the soil. Another related aspect is the limitation of methods for tracking of the strain applied. Till date this is a tedious job with successful efforts been made mostly under controlled conditions. Not just their persistence, their (strain specific) efficacy can also not be monitored with confidence under in vivo conditions. Besides, the issue of culturability of microorganisms is a Herculean task with majority being as yet uncultured. Hence, we tend to miss out on the potential of vast population that has not been cultured yet. Apart from these issues, as these amendments are biological entities, their response to various stressors and behaviour under different environmental fluctuations in vivo is less understood.

Attempts have been made to overcome some of the above-mentioned limitations. One important approach that is currently followed by many researchers is the selection of indigenous strains for application as bioinoculant. This is believed to not just ascertain survivability in the rhizosphere but also its efficacy. A strain isolated from the system is assumed to be unidentified as “foreign”, and thus will experience minimum competition from resident microflora.

The traditional method to track bioinoculant strains has been application of antibiotic mutant strains of the same. Such a release has mostly been attempted in pot experiments (Bolstridge et al. 2009; Compeau et al. 1988; Egamberdieva et al. 2011; Loper et al. 1984) with a few studies performed in field (Hynes et al. 2001; Liu et al. 1992). Molecular tools have come to the rescue of tracking of applied strains. Identification of sequences unique to the strain of interest has been performed by the technique of Random Amplified Polymorphic DNA (RAPD) PCR. Subsequently, these identified sequences can be utilized through the method of SCAR markers, to quantifiably assess the dynamics of strains in soil. However, there are limited reports on successful application of this approach, most of them being restricted to *Trichoderma* sp. (Parmar et al. 2015; Ruano-Rosa et al. 2014; Rubio et al. 2005). Some of the other microorganisms that have been tracked include *Aspergillus* sp. (Devi et al. 2013), *Pseudomonas fluorescens* (von Felten et al. 2010; Pujol et al. 2005) and *Rhizoctonia solani* (Ganeshamoorthi and Dubey 2013). Hence, further efforts are required in the area.

11.4 Rhizosphere Microbiome Engineering

In view of the limitations of applying single or multiple strains forming a consortium for plant growth promotion in agriculture, a relatively risk-free path is considering the meta-organism or holobiont, and attempting to optimize the entire framework rather than individually doing so. The objective of engineering the rhizospheric microbiome is to shape it up in a direction to favour the functions of plant. Such functions could be anything from enhancement of crop yield, mitigation of stresses, reduction in disease susceptibility, etc., depending on individual requirements (Ryan et al. 2009; Zhang et al. 2015; Bakker et al. 2018). Two major techniques employed for engineering rhizospheric microbiome have been (a) synthetic microbial communities, and (b) host-mediated artificial selection (Ahkami et al. 2017; Dessaux et al. 2016; Foo et al. 2017) (Fig. 11.1).

The first step to meet such a goal is to identify the critical microbial components for designing of a minimal or core microbiome. Core microbiome is defined as the collection of microbial taxa consistently associated with a healthy plant. It is in fact a pre-requisite for attempting rhizosphere microbiome engineering and a priority area as specified by Busby et al. (2017). With advancement in techniques, especially with popularity of “omics”-based methodologies, reports have started coming in on identification of core or minimal microbiome for plants like *Arabidopsis* (Bulgarelli et al. 2012; Lundberg et al. 2012), beech (Colin et al. 2017), sugarcane (Yeoh et al. 2016; Hamonts et al. 2018), maize (Walters et al. 2018), *Salvia miltiorrhiza* (Chen et al. 2018), potato (Pfeiffer et al. 2016), poplar (Beckers et al. 2017), etc.

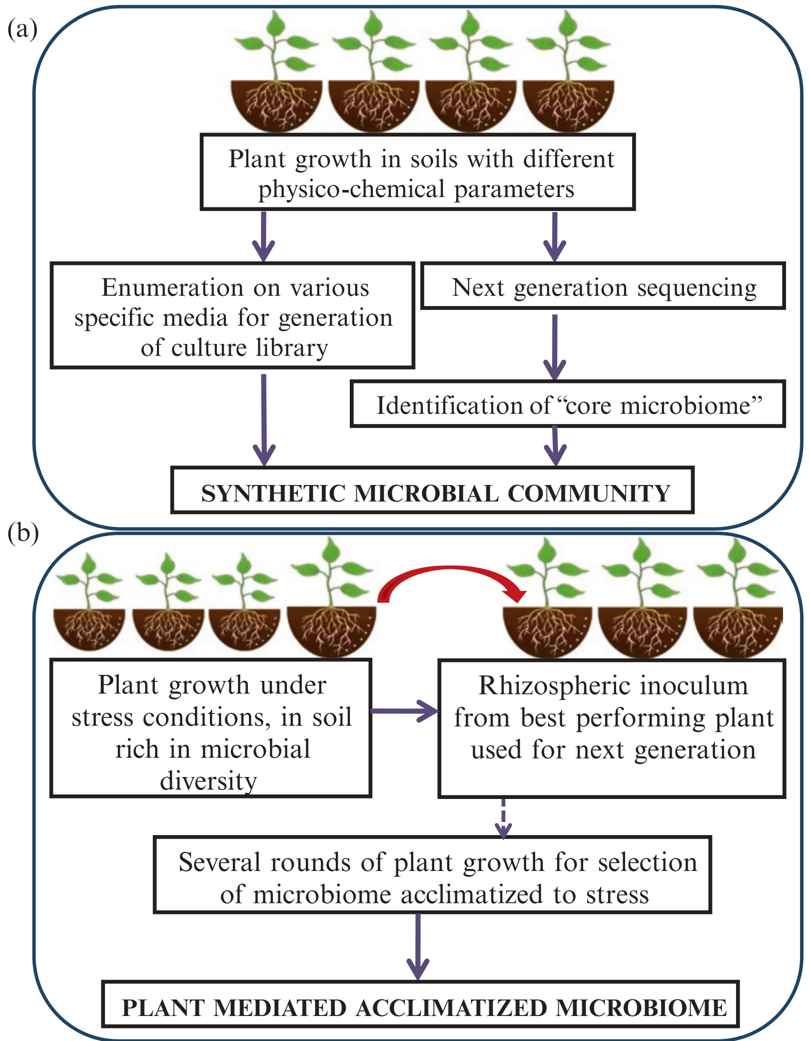

Fig. 11.1 Approaches to engineer the rhizospheric microbiome: (**a**) generation of synthetic microbial communities (SMC) and (**b**) plant-mediated microbiome selection

11.4.1 Synthetic Microbial Community (SMC) or Synthetic Community (SynCom)

Mixed cultures have been used for biotechnological applications since decades. This includes a mix of microorganisms with regular functions, which may or may not be specifically known. A synthetic microbial community (SMC), on the other hand, is designed by strategically selecting its components to perform a definite task, and complementing the function of the community in totality. An important

feature of SMC is its defined structure with less complexity as compared to the natural microbiome (Grosskopf and Soyer 2014).

It has been well established that attaining "naturalness" is difficult, especially so in agriculture. Imitating the complexity of nature in the rhizosphere can only be attained upon thorough understanding of the interwoven interactions between different functional groups, which has till date not been achieved. This is despite technological developments by leaps and bounds. Hence, the only other alternative is to retain the indispensable, key microbial members in contributing to a functionally stable microbial community, which is much simpler than the "natural" community. There should, however, be least compromise with the performance of such an artificially generated microbiome in terms of its impact on plant growth promotion. Principles of designing a SMC have been extensively discussed (Busby et al. 2017; Johns et al. 2016; Johnson et al. 2015; Qin et al. 2016; Sivasubramaniam and Franks 2016; Vorholt et al. 2017; Widder et al. 2016; Williams and Lenton 2007). SMCs have application in various biotechnological processes including microbial fuel cells, treatment of wastewater, etc. However, in agriculture, such communities have been designed and tested only recently, mostly under gnotobiotic conditions in the experimental model plant *Arabidopsis thaliana* (Bodenhausen et al. 2013; Bai et al. 2015; Lebeis et al. 2015; Wallenstein 2017). While Lebeis et al. (2015) designed a SMC with 38 strains and reported the significance of salicylic acid in assemblage of rhizospheric microbiome in *A. thaliana*, Bodenhausen et al. (2013, 2014) used 7 strains from laboratory culture collection to constitute a less complex SMC for the same host. A couple of studies have also successfully reported SMCs to be functional in plants like maize (Armanhi et al. 2018) and tomato (Hu et al. 2016). In fact, Armanhi et al. (2018) assembled SMC comprising of 17 isolates from sugarcane rhizosphere, and this was successfully applied to maize. A list of reports on generation of SMC, with their composition and respective plant system, has been compiled in Table 11.1.

For designing of a synthetic community, besides an extensive study on characterization of the host's core microbiome, an elaborate culture collection is a prerequisite. Such a generation of resource can be tedious and time-consuming. For selecting the building blocks of SMC, two types of interactions are required to be assessed, viz. microbe-microbe and plant-microbe. The former binary association assessment indicates beneficial or inhibitory relationship between microbial members, while the latter gives an idea of their impact on plant phenotypes. SMC could be of low complexity, which is easier to handle with the function of every component being well defined. In such a case, each strain can be studied for their persistence and function. However, a simple community might bypass certain important associations or inter-relationships, which is desirable for optimum functioning of the host. A highly complex SMC, on the other hand, has its own sets of limitations and advantages. It has a better chance of associations being intact, hence enhanced efficiency and competence. Robustness and stability of the SMC generated needs to be monitored before bringing it into application. Stability can be ascertained only when there exists strong synergism between members. Said and Or (2017) suggest renewal of the assembled community by replacing cells so as to avoid development of "cheaters" through mutations or horizontal gene transfers. In fact, they propose

Table 11.1 Compilation of reports on generation of synthetic microbial communities for enhancement of plant parameter(s)

Plant System	Members of SMC	References
Arabidopsis	*Arthrobacter* sp., *Methylobacterium extorquens*, *Methylobacterium radiotolerans*, *Rhodococcus* sp., *Sphingomonas* sp., *Sphingomonas phyllosphaerae*, *Variovorax* sp.(phyllospheric SMC)	Bodenhausen et al. (2014)
	Leaf SynCom: 218 leaf-derived bacteria, rhizospheric SynCom: 188 members (158 root-derived, 30 soil-derived bacteria)	Bai et al. (2015)
	38 strains	Lebeis et al. (2015)
	Strains of *Microbacterium* sp., *Stenotrophomonas* sp., *Xanthomonas* sp.	Berendsen et al. (2018)
Tomato	48 communities using 8 genotypes of *Pseudomonas fluorescens*	Djavaheri et al. (2012) and Hu et al. (2016)
Maize	*Chryseobacterium indologenes*, *Curtobacterium pusillum, Enterobacter cloacae, Herbaspirillum frisingense, Ochrobactrum pituitosum, Pseudomonas putida*, *Stenotrophomonas maltophilia*	Niu et al. (2017)
	Strains of *Asticcacaulis, Bosea, Burkholderia, Dyella, Chitinophaga, Ensifer, Enterobacter, Lysobacter, Microbacterium, Pantoea, Pedobacter, Pseudoxanthomonas, Rhizobium, Sphingomonas, Stenotrophomonas*, and 2 unknown genera of Comamonadaceae and Streptomycetaceae	Armanhi et al. (2018)
Poplar	Strains of *Acinetobacter calcoaceticus, Burkholderia* sp., *Burkholderia vietnamiensis, Curtobacterium* sp., *Enterobacter asburiae, Pseudomonas* sp., *Rahnella* sp., *Rhizobium tropici, Rhodotorula graminis, Sphingomonas yanoikuyae*	Khan et al. (2016)

introducing redundancies for stability, in case there are such disturbances leading to an adverse effect on the functioning. Synthetic biology plays a very important role in designing SMC so as to dictate the members to focus their function towards benefitting the host (Brenner et al. 2008; del Carmen Orozco-Mosqueda et al. 2018). Attempts have been made to develop algorithm for assemblage of robust SMCs (Eng and Borenstein 2016).

A simple SMC comprising of 7 strains was designed by Niu et al. (2017) for maize wherein *Enterobacter cloacae* was identified as the keystone species. The choice of strain was dependent on selection by the plant. The SMC was successful in inhibiting the phytopathogen *Fusarium verticillioides*. They even checked the dynamics of each strain by employing the method of enumeration on specific media.

In generation of an SMC for *Arabidopsis thaliana* for modified Pi content in plant, Paredes et al. (2018) began with in vitro screening of 440 bacterial isolates. Further they were categorized into “functional bacterial blocks” on the basis of binary associations (isolate + host). A set of 14 partially overlapping SMCs were

generated, each with two bacterial blocks. These were tested with plant for their impact on different plant parameters.

Moronta-Barrios et al. (2018) attempted to assemble an endophytic SMC for rice, comprising of 10 isolates. A total of 87 endophytic isolates were screened for their PGP properties. Inoculation of rice seedlings was done in Hoagland solution with a simplified SMC assembled from 10 isolates. Redundancy in terms of functions of bacterial members was avoided, together with exclusion of isolates producing antibacterial compounds under in vitro conditions. 16S rRNA marker was used to track each strain for its colonization ability. Only four out of the ten isolates could be detected after 30 days of plant growth.

11.4.2 Plant-Mediated Microbiome Selection

For enhancement of plant's fitness, targeted evolution of microbiomes is a novel approach. As there is an artificial or indirect selection of the microbiome by the plant, the approach has been coined as host-mediated microbiome selection (Mueller and Sachs 2015). Such a selection is directed towards enhancing the performance of the plant. The microbiomes are not measured directly, but evaluated indirectly by measuring plant's performance. Such an approach involves combined evolutionary and ecological changes to the microbiome.

The enhancement of performance can be through any trait including growth parameters, resistance to pathogen or ability to mitigate stresses. The usual method of such a selection is to establish multi-generational plant growth cycles. The rhizospheric microbiome from the best performing plants (in terms of the selected parameter to be assessed) is applied to the subsequent generation as inoculum. Hence, it is the plant trait that is used to evolve the microbiome. By this approach it is only the microbial component of the holobiont that is selected, restricting the evolution of the plant by using the same genotype for all the generations. Structure and function of microbiome that have been thus selected can be analysed to compare it with the core microbiome.

This is different from generation of synthetic microbial communities in the sense that SMCs are constituted exclusively of culturable isolates, while such an approach brings together both culturable and as-yet-unculturable fraction. But this leads to the advantage that the composition of SMC is being better controlled. Table 11.2 lists the other similarities and differences between approaches I and II.

The first report to select microbiome artificially on the basis of phenotypic trait of plant was by Swenson et al. (2000). With the model plant, *Arabidopsis thaliana*, a microcosm experiment was set with repeated generations of plant growth. The offspring generation was inoculated with the soil slurry formed from "best-performing" microcosm of preceding generation (parameter: dry weight). Two sets, differing in the order of magnitude of the inoculum, were run parallel for several generations. This was the first study to successfully report artificial selection of micro-ecosystems, which was on the basis of plant phenotype. There was a gap of over a decade before the next report on such a multi-generation approach came up.

Table 11.2 Comparative analysis of two approaches of rhizospheric engineering, viz. generation of synthetic microbial communities, and host-mediated artificial selection of microbiome

Feature	Synthetic microbial communities	Host-mediated artificial selection
Process	Efficient strains artificially selected from culture collection	Microbes selected by the host in response to stresses/changes in external/internal environment
Culturability of members	Well-characterized culturable members	Comprises of both culturable and as-yet unculturables
Role of members	Interactions between members and their potential effects on the host can be elucidated.	Individual roles of members can not be ascertained in view of the complexity
Controllable	Easy to control as defined strains make up the community	Difficult to control as large number of unidentified microbes constitute the community
Survival and competence	Chances of survival is lesser due to limited interactions between strains, greater possibility of being out competed by indigenous rhizospheric microbial community	Greater chance of survivability and competence as the simplified microbiome is selected by the plant and is well adapted to the habitat
Easy of generation	Constitution easier with individual stocks of strains	Selection over multiple generations required, time taking
Reproducibility in generation	Highly reproducible	Low to no reproducibility
Storage	Easy as individual members can be stored as glycerol stocks for long term.	Tedious to store the complex microbiome comprising of both culturables and as-yet unculturables

Using the same model plant, Panke-Buisse et al. (2015) set repeated generations of growth for selection of microbiome for early and late flowering. From the tenth generation, they inoculated three different genotypes of the crop and another plant, *Brassica rapa*. They could successfully demonstrate a change in flowering time upon such an amendment even in the related crucifer. They went on further to identify the microbiome thus generated by Illumina. A core microbiome could be established by comparing Operational Taxonomic Units observed across treatments and control. Rhizospheric microbiomes associated with early and late flowering were distinct in terms of the diversity observed. A major difference between approaches followed by Swenson et al. (2000) and Panke-Buisse et al. (2015) was in terms of the starter microbiome employed. Starter microbiome used by Panke-Buisse et al. (2015) was a mix of soils from varied habitats including arable land, forest, grassland, etc. This was done to have a rich diversity in the starter microbiome, and was in contrast to Swenson et al. (2000) who used only a single source for soil used in the study. It is debatable whether bringing together multiple sources of microbiome is at all advantageous over single source as it is argued that the latter might already possess community modules, the intactness of which is desirable.

It was simultaneously that Rillig et al. (2015) introduced the concept of "community coalescence", which involves bringing together separate microbiomes, so as

to facilitate development of newer networks. This phenomenon occurs naturally as well, e.g. endophytes in seeds mixing with resident soil microflora. They put forth this approach as a promising tool for rhizosphere engineering in sustainable agriculture (Rillig et al. 2016a, b). In fact, in both papers (Swenson et al. 2000; Panke-Buisse et al. 2015), a milder form of unintentional community coalescence occurred when replicates of best performing pots were mixed to generate inoculum for subsequent generation.

In contrast to reports of enhanced plant growth parameter with selected microbiome, the study by Yergeau et al. (2015) brought forth a different set of data. They worked towards looking at the extent to which rhizospheric microbiome affects plant phenotypes in willow. After one generation of growth of willows in unmodified soil, the soil was used as an inoculum for second generation of plant growth post gamma-irradiation of soil. They could demonstrate the strong effect of willow on its rhizospheric community, with the selected microbiome exerting only a short-term effect, and then converging to the microbiome at T0. Their hypothesis of a plant inoculated with rhizospheric microbiome of a large first-generation willow to exhibit better phenotype could not be proven.

The method of Swenson et al. (2000) was later evolved by adopting a "differential microbiome-propagation method", in order to confer salt tolerance to the grass *Brachypodium distachyon* (Mueller et al. 2016). They selected a sub-microbiome by fractionating bacterial and viral members using filters (excluding fungal, protozoan and other members). Non-evolving surface sterilized seeds were inoculated with rhizosphere bacteria harvested from the plants exhibiting the best trait. Besides, with every generation ramping of salt stress was done.

Yuan et al. (2016) followed a different approach in first generating an SMC by inoculating sample of rhizospheric soil of *Suaeda salsa*. Subsequently, the SMC was acclimatized under saline conditions by amending with 2% sea salt solution. The acclimatized microbiome could successfully improve plant's ability to tolerate salt stress. In a multi-generation experiment with *Brassica rapa*, Lau and Lennon (2012) observed a positive response of the rhizospheric community to multi-generational soil-moisture treatments.

11.5 Challenges in Rhizosphere Microbiome Engineering

11.5.1 Domestication of Higher Plants Is Time-Consuming

Most of the studies on plant-mediated selection and SMC generation have been successfully demonstrated on the model crop *Arabidopsis*. Multi-generational domestication of microbiome for other plants with larger growth cycle is time-consuming and requires controlled conditions so as to avoid environmental fluctuations. This is probably one of the major reasons why there still exists only a handful documentation of successful generation of plant-mediated microbiome.

11.5.2 Thorough Understanding of "Core Microbiome"

Extensive studies need to be performed to first identify the minimal or core microbiome before attempting its engineering. The metagenomics approach to achieve this has picked up in recent years. Generation of sequence data is not sufficient, especially when it comes to assembling SMC. A vast culture collection with well-characterized strains is also required. This has to be plant-specific. While the first set-up might not be cost- and time-effective, once established such a library can serve the scientific community in a large way.

11.5.3 Appropriate Plant Attributes for Selection

When performing selection of microbiome based on plant phenotype it is crucial to have an understanding of the phenotypic traits governed or influenced by the below-ground assembly of microbial members. Recently, Oyserman et al. (2018) proposed quantitative estimation of microbiome-associated phenotypes (MAPs), which have till date been assessed only qualitatively. They termed this as mathematical MAPs-first approach.

11.5.4 Propagation of Engineered Rhizospheric Community

A major challenge in the approach of plant-mediated selection of microbiome is the paucity of method to preserve the engineered microbiome for further application. This is relatively easier in case of SMC wherein the known and characterized strains can be stored as glycerol stocks and used for later constitution. Without pure cultures in hand in the selected microbiome, maintaining all the interactions between microbial members intact is a gruesome job. Only a single report has attempted rhizospheric microbiome preservation (Panke-Buisse et al. 2017). However, in this case only the culturable fraction was selected from the total microbiome after multi-generational plant growth, by cultivating them on four different media. Preservation of cultivated microbiomes was attempted by supplementation of glycerol, followed by freezing. They were then revived to assess the efficiency of media in preserving the microbiome, which exhibited the phenotypic trait for which it was selected (early flowering in this case). The preserved culturable fraction that was enumerated on 25% LB and 10% tryptic soy agar retained the desired trait.

11.5.5 Practical Issues of Application

There are certain points related to the technical issue of applying SMC to farmers' field in a vast manner, viz. the medium of growth of the strains, the duration of growth before generation of an SMC, the proportion of each strain to be mixed, and the tracking of individual strains in vivo. Similarly, the microbiome selected by the

plant also suffers from certain practical issues like, deciding the percent of inoculum to be applied, mode of application of the selected microbiome, determining the survival of the selected microbiome at regular intervals in vitro and in vivo. Thorough investigation needs to be performed for successful deployment of the techniques. This will also require knowledge and control on population growth under different environmental conditions (Johns et al. 2016). Also, the use of substrates as carriers for the stability of engineered community needs to be investigated.

11.5.6 Behaviour Under Natural Conditions

Both with SMC and plant-mediated selection of microbiome, their fate upon field application is hard to predict. Upon coalesce of applied microbial community with the resident microbiome, the outcome could be positive, negative or even neutral. Their interactions with the resident microbiome eventually decides their competence and efficiency in vivo. There is, by far, no measure of disruption to the engineered microbiome, other than by monitoring the plant phenotype for which the engineering of rhizosphere has been attempted. A failure in exerting the desired phenotype on the plant could be because of disintegration of the applied microbial community or because of environmental factors. Risk assessment of such an application is a pre-requisite before release in fields.

11.6 Conclusions and Future Perspectives

The rhizosphere is one of the most complex zones with a rich diversity of interactions found in the habitat, constituted of soil as the abiotic component, and roots together with associated microflora forming the biotic constituents. The concept of rhizosphere engineering by employing synthetic microbial communities and by plant-mediated selection seems to be promising with the proof of concept established; however, the two still need to be validated for a range of plant systems. Strategic domestication for mitigation of abiotic and biotic stresses needs to be attempted. Field studies on application of the generated microbiome are yet to be conducted by researchers. This is of utmost importance in deciding the feasibility of the technique. It is definitely a step towards sustainability in agriculture. From the reports presented, it is clear that bringing such a technology to the field requires expertise from a wide array of scientists including microbial and plant physiologists, ecologists, specialists in plant-microbe interactions, agronomists, bioinformaticians, systems biologists and more.

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12 Dynamics of Plant Microbiome and Its Effect on the Plant Traits

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Abstract

Plants host a plethora of complex microbial communities in and on their surfaces designated as plant microbiome. The plant microbiome symbolizes the collective communities of microbes, their (meta) genomes and their interactions (mutualism-antagonism continuum) in a particular environment. The cross-talk between plant microbiome plays an important function in the performance of plant and is hot topic for research in biology. Plant microbiome endows the plant with resistance to biotic and abiotic factors, promotes plant growth and enriches the soil associated with the plant. The plant trait expression is regulated by the orchestrated effect of plant as well as microbial genes. Therefore, there is an urgent need to explore the diversity and the functionally potential of microbial communities. However, a big challenge in the present scenario is to widen technologies to improve agricultural management, e.g. plant growth promotion, biocontrol and bioremediation. Recent advances in sequencing technologies and multi-omics approaches integrate the studies on plant-microbe interactions, which gives an insight about what's happening in real-time within the cells. Metatranscriptomics and metaproteomics have come up as a holistic approach that give a picture of major metabolic pathways and the plant-associated interactions. These technologies clearly depict which functional microbial communities are dominant in crop plants and under different environmental conditions. The integration of various computational tools helps to decode the functions of proteins, individual signal molecules and gene cascades, with respect to their pathways.

Keywords

Plants · microbes · Microbiome · Plant-microbe interactions · Metagenomics · Plant traits

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12.1 Introduction

Both biotic and the abiotic systems cannot function without interacting with their microbiome, be it an animal, plant or our planet earth. Most of the bio-geothermal cycles are run by microbes and this makes life on earth possible (Boundy-Mills 2006). Microbes that are associated with the plant, animal and human systems include bacteria, archaea and fungi. They play numerous roles in maintaining health and growth of organisms, by designing behavioural strategies for avoiding or removing pathogens. Emphasis on studying the microbial function and community structure is a growing area of research (Bahrndorff et al. 2016; Ezenwa et al. 2012; Liu et al. 2012).

The importance of microbes for plants has been recognized hundreds of years ago and is credited to Lorenz Hiltner (1904). He developed many techniques to study plant-microbe interaction by combination of microscopy and analytical tools. His research is considered as a milestone in the field of plant-microbe interaction (Caporaso et al. 2012; Jansson et al. 2012). The cognisance reveals a very close symbiotic relationship between plants and their associated microbiomes. There is diversity in plant microbiomics, both at structural and functional level. Plants dock microbes within specific habitats, which can be classified as rhizosphere, the area around roots (Berendsen et al. 2012); phyllosphere, the area around above-ground parts (Vorholt 2012); and endosphere, the internal tissues (Hardoim et al. 2015).

Lorenz Hiltner in 1904 was the first to observe that soil surrounding the roots of the plants are loaded with microorganisms, as compared to soil distant from the root and called this area the rhizosphere. The rhizosphere is considered to be a complex system, where the interaction between the plant and the microbes occurs, playing a significant role in plant health. The plant and soil microbial community modulates the plant nutrition uptake and growth rate, resistance to environmental stress factors, plant survival and sustenance (Mendes et al. 2013). Therefore, the overall fitness of the plant depends on its associated microbiota, which together forms the plant 'holobiont'. The term holobiont was coined by Lynn Margulis in 1991 in the book *Symbiosis as a Source of Evolutionary Innovation* for the assemblages of different species (eukaryotes and prokaryotes) that form one ecological unit. The associated bacterial and fungal communities within a given ecological niche were then started being called the 'microbiome' in analogy to genome (Richardson 2017).

Although rhizosphere is the extensively studied plant microbial niche till date, in the recent years, the study on the microbiome of phyllosphere is also increasing. The phyllosphere is also termed as pervasive global habitat of diverse microbial communities. It is an interface between the plant parts which are above the ground and the atmosphere. The microbial communities from phyllosphere were found potentially active to influence plant biogeography and ecosystem function. These communities also influence the fitness and function of their hosts in the same way the rhizospheric community does (Kembel et al. 2014). The abundance of phyllospheric communities is estimated to be more than 10^{26} in a billion square km leaf surface area worldwide. These aerial inhabitants represent one of the largest biological interfaces on earth. They caper roles in CO_2 fixation, molecular O_2 release,

nitrogen cycle and primary biomass production (Delmotte et al. 2009). A square cm of leaf is estimated to have 10^6–10^7 inhabitants (Lindow and Brandl 2003).

Microbes not only inhabit outer surfaces, but also live deep inside the cells and tissues. These are termed 'the endophytes'. The first proof of existence of endophytes was given by Victor Galippe in the year 1887. Hallmann and co-workers in the year 1997 gave practical description of endophytes. Endophytes play both helpful and harmful roles for their hosts. They can be both mutualists and antagonists depending on the prevailing conditions (Hardoim et al. 2015). The helpful ones produce numerous secondary metabolites, proteins, enzymes, small RNA, etc. that promote plant growth and help them adapt better to their surroundings (Nair and Padmavathy 2014). Endophytes have been exploited as sources of many antimicrobial compounds. Highly diverse endophytes of medicinal plants have been identified as seed germination helpers and oxidative stress relievers. A large number of plant growth-promoting endophytes are being isolated and widely used to improve quantitative and qualitative yield of plants (Santoyo et al. 2016; Khan et al. 2017).

Microbial community structure changes when we move from rhizosphere to the endosphere. However, the positive impacts obscure the negatives in all areas. There is a wide scope to extensively utilize these microbial communities for the betterment of environment, as a whole. As on today, the presence of microbiome associated with almost all ecological niches is an established fact and emphasis is on the role they play in the niche they reside in (Fig. 12.1).

12.2 Effect of Microbiome on Plant Traits

The plants interact with the environment through its underground part – the roots – and aerial parts, stem, leaves and fruits. All these parts have a vast diversity of associated microflora. Therefore, it becomes important to unravel diversity of microbial communities and how they interact with the plants and regulated plant traits. The diverse microbial community associated with plants, the plant microbiome, shows a similar impact on plants, as gut and skin microbiome have on human health (Berendsen et al. 2012). Any imbalance or disturbances in the microbial species can result in disease outbreaks, in both plants as well as animals. Therefore, there is a need to maintain the healthy state of the host and suppressing the disease-causing pathogens of the host's native microbiome. Broadly, plant traits can be divided into four categories – morphology (leaf and root anatomy, length or shape), reproduction (clonal growth, fruit dimensions, number of seed, reproduction type, etc.), phenology (flowering age and time, leaflets growing time, etc.) and ecology (physical environment conditions, biotic and abiotic stress tolerance, etc.).

The microbiome has been found to have several noticeable deep effects on seedling vigour, seed germination, development of the innate immune system and nutrition (Mendes et al. 2013; Berg et al. 2014a, b; Schikora et al. 2016). By introducing bacteria into plant seeds, plant traits can be enhanced. Mitter and co-workers (2017) proved that in maize seeds, co-inoculation with *Paraburkholderia phytofirmans* Ps JN can improve growth and development as compared with non-treated samples.

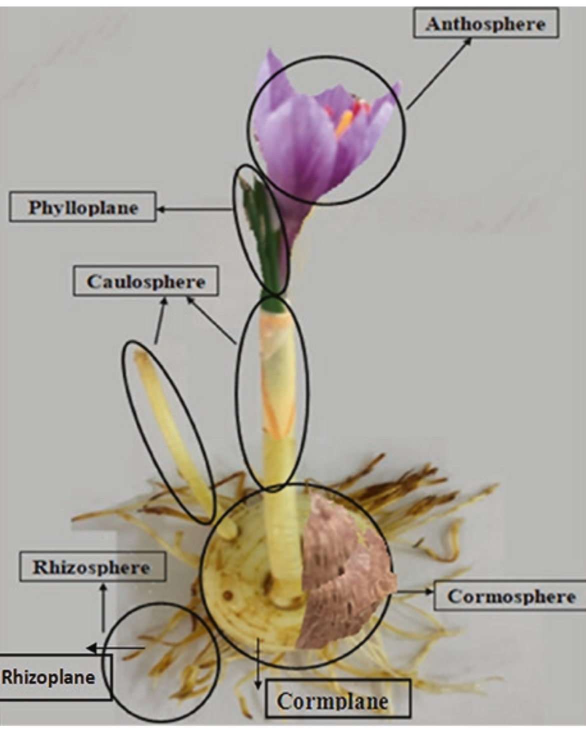

Fig. 12.1 The diagrammatic representation of plant parts where microbial interactions take place

This gives an idea about altering microbiome to modulate the behaviour of host as per the requirement.

Different plant functional traits of leaf and the roots traits have been measured with response to the microbial interactions and manipulations. The plant-associated microbes mediate plant traits either by synthesizing biologically active compounds, which provide novel biochemical competence, and/or by altering the plant existing metabolic pathways (Friesen et al. 2011). In *Brassica rapa*, it was observed that by altering the composition of the below-ground part associated microbial communities, the plant grew smaller in size with lower chlorophyll content and less number of flowers, compared with plant populations that were grown along with complex and diverse soil microbiome (Lau and Lennon 2011). Inoculating strawberry plant with bacteria *Bacillus amylolequifaciens* and *Paraburkholderia fungorum* significantly shows enhanced growth in leaf length/number and shoot/root dry weight (Rahman et al. 2018).

When the roots are young, the rate of nitrate, potassium and phosphate uptake is more. The dry matter and NPK accumulate at a fast pace and thus crop yield will be high. The need is to look for such microbiome that changes plant root trait. Okon and Kapulnik (1986) isolated several *Azospirillum* strains which influence morphological changes in roots in wheat, sorghum, corn and setaria. The age of bacterial inocula and its concentration differentially affect the surface area and length of roots. The number of root hairs, branches of root hair and lateral roots increases by

inoculating bacteria during the first three weeks after germination of seeds. In the later stages of plant development, root biomass also increases (Okon and Kapulnik 1986; Fallik et al. 1994). The root microbiome also modulates cell division and differentiation in the primary root and lateral roots which influence root growth and development (Verbon and Liberman 2016).

The potential of plant-associated microbiome for flowering is also widely exploited. The soil microbiota has a great influence on the flowering phenology and fruit production. This has been proved in a wild relative of *Arabidopsis, Boechera stricta* (Lau and Lennon 2012; Wagner et al. 2014). In *Arabidopsis* also, the microbiome help in retaining functional traits of early flowering that were modified through artificial selection on flowering time (Panke-Buisse et al. 2017). Selected microbes associated with early and late flowering (EF and LF), when inoculated into soils of the novel plant *A. thaliana* hosts, show differences in the flowering times. Microbes associated with late flowering flowered 15–17% later with consequent increase in inflorescence biomass than plants inoculated with EF-linked microbiomes (Panke-Buisse et al. 2015).

The role of different bacteria and arbuscular mycorrhizae fungi (AMF) on clonal reproduction in plants has been widely studied in last few decades. Different species of AMF differentially alter clonal reproduction in many plants. Impacts of AMF on *Prunella vulgaris* show improved growth of ramets and clonal reproduction. AMF shows strong effects which could potentially affect size of population and variation of clonal reproduction in plant communities (Streitwolf-Engel et al. 2001). The symbiotic effects of the AMF are not only observed in colonized roots but also in the above-ground plant compartments, e.g. flowers, leaf and fruits. Studies have shown that AMF in combination with PGPB or alone positively affected fruit and flower size, due to accumulation of higher concentration of anthocyanin, sugars, ascorbic acid that resulted in earlier flowering & fruiting in strawberry. Co-inoculation with the AMF and PGPB shows distinguished results such as increased flower number and more fruit production along with larger size of fruit. In inoculated plants, the sugar concentration, folic and ascorbic acid concentration was also found to be higher compared with uninoculated plants (Lingua et al. 2013; Bona et al. 2015).

The plant growth-promoting bacteria (PGPB) have been known to elevate the nutritional and functional properties of plants like cumin and flax seed oil. The plants when inoculated with the PGPB (*Hymenobacter* sp., *Paenibacillus* sp. and *Streptomyces* sp.) modulate its nutritive properties that are reflected in increasing total polyphenols, flavonoids, careteniods and essential fatty acid content as well as resulted in greater antioxidant activity, compared with non-treated plant samples (Dimitrijevic et al. 2018). Plants like strawberry are known to exhibit different medicinal properties as it is a good source of natural antioxidants such as secondary metabolites, phenolic compounds and carotenoids and hence show free radical scavenging activities. The plants inoculated with friendly bacterial species enhance the production of total antioxidants compounds thereby enhancing functional properties of plants (Rahman et al. 2018). The characterization of the functional traits of culturable rice microbiome shows the potential for the production of IAA and N

fixation by different microbiome members suggesting their applications as plant growth promoters (Venkatachalam et al. 2016).

The plant microbiome co-operates among themselves and functions together to protect plant from stress. *Curvularia protuberate* and *Fusarium culmorum* show potential against crop heat and salt tolerance enhancement (Kandel 2016). Date palm (*Phoeix dactylifera)* endophytic bacteria *Enterobacter* sp. have been characterized and tested for the ability which help the plants to grow under saline conditions, by producing stress-tolerant enzyme ACC deaminase (Yaish et al. 2015). Mycorrhizae and PGPB help in improving crop productivity under unfavourable environment by increasing uptake of nutrients (e.g. N and P) and increase surface area of roots. As the surface area of roots increases, there is an increase in the availability of nutrients for plant consumption (Nadeem et al. 2014). Proteobacteria are reported to provide protection against nematodes and stress resistance to plants (Allen et al. 2015; Cao et al. 2015).

To maintain healthy ecological conditions, microbes help plants in remediating pollutants from soil as well to sustain healthy ecological balance. *H.cannabinus* maintains a core root microbiome consisting of Enterobacteriaceae, Pseudomonadaceae and Comamonadaceae when it grows in area of metal pollution (Chen et al. 2018). Plants also scavenge air pollutants significantly from atmosphere through their above-ground parts. Plant-associated microbiomes degrade, detoxify and sequester the pollutants to clean the environment (Stevens 2016). The inoculation of AMF in legume tree elicited the phytoremediation of the soil polluted with lead, and rhizoremediation helps maintaining the natural flora and fauna for ecological sustenance (Yang et al. 2016) (Table 12.1).

12.3 Approaches to Study the Plant Microbiome

As humans, plants have also been recently categorized as meta-organisms that harbour a distinct microbiome and live in a symbiotic relationship with its associated microflora (Berg et al. 2013), thus making its study a curious area of exploration.

The study of a plants' microbiome involves two parts: studying the community structure and the community function.

12.3.1 Studying the Community Structure

It includes studying the composition of a plants' microbiome in terms of its microbial diversity and species richness, i.e. who is there?

As already discussed, the plant harbours a diverse range of microbial communities that exist in an interactive relationship with each other and with the plant. It has been challenging to completely define the composition of plant microbiome. However, the advent of various approaches has made this path easy. Cultivation dependent and independent are the two approaches available to determine the makeup of a plant microbiome. Conventionally, evaluation of microbial diversity

Table 12.1 Effect of various bioelicitors on traits of the host plant

Microorganisms	Host plant	Effect on the plant traits	References
Pseudomonas strain	*Fragaria x ananassa var. Selva* (Strawberry)	Increased plant growth and nutrition, increases anthocyanin concentration	Lingua et al. (2013)
Pseudomonas sp., *Bacillus subtilis* and *Pantoea* sp.	*Crocus sativus* L	Increased weight in cormlets	Parray et al. (2013)
Pseudomonas putida, Azospirilium, Azotobacter	Artichoke (Cynara scolymus)	Increase in the radical length, shoot length/weight, decreased mean time of germination	Jahanian et al. (2012)
Phyllobacterium brassicacearum strain STM196	*Arabidopsis*	Decreased leaf transpiration as a result of enhanced ABA content	Bresson et al. (2013)
Arbuscular Mycorrhizal Fungi (AMF)	*Crocus sativus* L.	Increase in weight and no. of cormlets, plant height	Lone et al. (2016)
Glomus intraradices, *Glomus mosseae*	*Solanum tuberosum* L. (potato)	Higher level of metabolites and mineral nutrition	Shuab et al. (2017)
Species of *Glomus genus*	*Allium cepa* (Onion)	Increase in chlorophyll content, growth morphology, fresh and dry weight of matter	Shuab et al. (2014)
P. putida H-2–3	Soybean	Improved plant growth as a result of gibberellins secretion	Kang et al. (2014)
AMF+Psedomonas strains	Strawberry	Increase in production of flower and fruit, with larger fruit size	Bona et al. (2015)
Rhizobium leguminosarum (LR-30), *Mesorhizobium ciceri* (CR-30 and CR-39), Rhizobium phaseoli (MR-2)	Wheat	Improved plant growth, biomass and drought tolerance index due to IAA produced	Hussain et al. (2014)

from different parts of plant was based on the use of cultivation-/culture-dependent approach that proceeded by initially isolating the microbes from their host plant by cultivating them in vitro on different nutrient media followed by their identification and characterization. It relied upon the difference in colony morphology, size, number of colony forming units (CFUs) and genotype (small subunit rRNA gene/Inter Spacer Transcribed region) for diversity studies. Cultivation-dependent approach could draw valid conclusions regarding the specific microbes but at the same time limited the unravelling of the community diversity due to the fact that just 0.001–1% of the microbial diversity can be cultivated by routine laboratory techniques (Torsvik and Øvreås 2002; Alain and Querellou 2009). The knowledge that about 99% of microbes circumvent this routine laboratory cultivation led to the advent of the cultivation-independent approach for cataloguing the microbes directly from the

source. Now a plethora of culture-independent approaches is available to study the composition and function of plant microbiome. This is done by isolating the DNA, sequencing the reads and analysing them by bioinformatic analysis.

12.3.2 Studying the Community Function

It covers the functional relationship between the plant and its native microbiota that modulates the growth and development of its host plant, i.e. what are they doing there?

Focus of microbiome research has shifted from just studying the community structure to linking the community structure with its function. One way of such study is to analyse the fluctuating plant genetic expression in presence of the variable environmental conditions. This basically includes studying the genome, metabolites, protein sequences, effect of the microbial communities on the plant genotype as well as phenotype.

The methodology for studying the plant microbiome community structure and function includes:

- DNA isolation and purification
- Sequencing and bioinformatic analysis

The methods of DNA isolation from plants differ with the site or the microenvironment. Considering the plant microenvironment, the isolation can be carried out in different manners. The exterior, the interior, above- and below-ground parts of a plant comprise a complex ecosystem, which in the recent years has got evolved in the form of a collective term called the 'phytosphere'. Further, the phytosphere is sub-divided into various small microhabitats like the endorhiza (root), spermosphere (seeds), anthosphere (flower), carposphere (fruit) (Fürnkranz et al. 2012) and cormosphere (Ambardar et al. 2014).

However, a plant has been compartmentalized based on the tissue environment – endosphere (inner tissue) and ectosphere (outer surface) (Ryan et al. 2008). These microhabitats present in the plant phytosphere comprise of a diverse environments in terms of their physical, chemical and biological environment. Such differences require the need to adopt different methods of sampling and nucleic acid (DNA) isolation depending upon the microbial community in focus and the extraction method in use.

DNA isolation majorly can be divided into two parts. The first is from the surface of the plants that acts as the exophytic environment for various microbial inhabitants forming the plant exomicrobiome and the second is the microenvironment inside the plant tissues colonizing a large plethora of microbial endophytes thus forming an endomicrobiome environment.

The exomicrobiome encompasses a vast range of microbes inhabiting the surface or the external region of the plant directly in contact with the external environment.

This includes the phyllosphere (surface of leaf) (Vorholt 2012), rhizoplane (root-soil interface) and rhizospheric soil (McNear Jr. 2013).

The basic differences in the DNA isolation from the exospheric and endospheric part lie in the fact that while targeting the exospheric microflora, we need not surface sterilize the sample which may otherwise lead to loss of the microbial community in focus. The microbial genome extracted from the exosphere is much lower in biomass relative to the total plant genome thereby creating more chances of having higher amounts of plant DNA/RNA in the extraction sample. However while studying about the rhizosphere microbiome, the quality and quantity of the rhizospheric soil can get hampered due to handling procedures among which a large amount of bulk soil can get transferred along, thereby masking the analysis of the rhizospheric community which needs to be taken care of.

Endomicrobiome is the environment composed of the microbes that can be bacteria or fungi which inhabit inside different tissues of the plant such as the interior of roots, leaves, flowers, seeds, etc.; these organisms have been referred to as endophytes (de Bary 1866). A common endophyte isolation protocol begins with the surface sterilization of the plant tissue surfaces to avoid the inclusion of the surface microflora. The efficiency of the protocol relies on the fact that it must exclude microbes residing on the surfaces of host plants such as fungi and bacteria on the lipophilic waxy plant cuticle surface (Müller and Riederer 2005). On the aerial surface, the microorganisms are more in number compared with plant tissues (Lindow and Brandl 2003); this emphasizes on the importance of extraneous DNA removal. Certain methods incorporate the use of aseptic peeling of tissue surfaces for sterilizations which is not possible for every plant tissue. The processes of cell disruption are standardized for various plant microbiomes and are permutations and combinations of thermal, chemical, physical and enzymatic lysis. Physical treatments such as bead-beating homogenization, sonification, vortexing (Steffan et al. 1988; Miller et al. 1999; Niemi et al. 2001; Miller 2001) and thermal shock (Tsai et al. 1991; Moré et al. 1994; Porteous et al. 1997; Orsini and Romano-Spica 2001) destroy the cell structure creating an access to the whole microbial community, including microbes hidden deep within. The physical method requires preliminary crushing and grinding of the material allowing the extraction or the lysis buffer to access the cells properly. Sodium dodecyl sulphate (SDS) is commonly chemical used, which is an anionic detergent, which dissolves the hydrophobic part of cell membranes. Detergents have often been used in combination with chelating agents like EDTA, Chelex 100 (Robe et al. 2003) and different buffers like Tris and sodium phosphate (Krsek and Wellington 1999) along with heat treatment. Cetyltrimethyl-ammonium bromide (CTAB) forms insoluble complexes with denatured proteins, polysaccharides and cell debris (Saano et al. 1995) and is also used for cell lysis. Enzymatic methods involve the digestion of samples by different enzymes affecting the DNA in the least way possible and particularly used in the case of Gram-positive bacteria that hold resistance to physical and chemical isolation methods (Tsai et al. 1991; Tebbe and Vahjen 1993; Zhou et al. 1996; Niemi et al. 2001).

High-quality DNA is extremely important for the accuracy of the followed procedure. The presence of proteins or polysaccharides may reduce the efficiency of

the Taq DNA polymerase, thus compromising the end products to be further analysed, thus making it a necessity to concentrate the DNA and remove any prevailing contaminants (Nunes et al. 2011). Ion-exchange chromatography, agarose, Sephadex gel filtration and PVPP/PVP gel electrophoresis are used for further purification of DNA after preliminary extraction (Cullen and Hirsch 1998). As this method consumes a lot of time, faster alternatives have been developed that include the DNA extraction and purification kits that can process numerous samples and result in a relatively pure DNA within a short span. Cesium chloride density gradient centrifugation has often been used to purify high-quality DNA (Robe et al. 2003). Further purity of the concentrated DNA can be detected using spectrophotometer-based analysis. DNA absorbs UV light maximally at 260 nm and proteins absorb light maximally at 280 nm. This means that DNA absorbs light at 260 nm 1.8 times more strongly than at 280 nm. If there is protein contaminants in the purified DNA, the absorbance at 280 nm increases. In such case, equal volumes of phenol/chloroform can be added for removal of protein contamination. Then the purified DNA is sequenced and analysed bioinformatically.

With the advent of DNA sequencing methods there is immense acceleration in the analysis of community structure and function from different plant parts. In the early 1970s, the sequencing methods started developing. Ray Wu at Cornell University developed location-specific primer extension strategy for determining DNA sequence (Wu and Taylor 1971). Maxam and Gilbert in 1977 developed chemical degradation method for sequencing. The first-generation sequencing method was developed by Sanger and co-workers in 1977. It was based on chain termination methods. First fully automated DNA sequencing method was developed by Dupont Genesis 2000 in 1987 (Prober et al. 1987). Then there was development of high-throughput sequencing technique called next-generation sequencing (NGS). Nowadays, sequencing is easier and faster compared with early times (Pettersson et al. 2009). NGS is widely used as this allows sequencing of millions of sequences simultaneously (Bentley et al. 2008).

The microbiome sequencing can be done by two approaches:

- Gene-targeted/amplicon microbiome sequencing
- Whole microbiome sequencing

Gene-Targeted/Amplicon Microbiome Sequencing

Gene-targeted microbiome sequencing is also called as amplicon sequencing. In this approach PCR amplification of specific target gene is done. Both community structure and function can be studied by this approach. To study community structure, there are phylogenetically important conserved sequences such as 16S rRNA, 18S rRNA, ITS, etc. Different sequence read comes from different organisms, so community structure can be easily studied as low abundant microbes can also be amplified (Kittelmann et al. 2015). Though amplicon sequencing gives good idea about community structure, it suffers limitation of PCR biasness (Pinto and Raskin 2012).

Whole Microbiome Sequencing

Whole microbiome constitutes all the available microorganisms in a particular habitat. Whole metagenomic sequencing (WMS) can be done to sequence whole microbiome, i.e. all the available species diversity at a particular place. It has multiple advantages over amplicon sequencing such as enhanced detection of bacterial species, better detection of diversity, enhanced prediction of genes, etc. (Ranjan et al. 2016).

To study community function, amplification of specific genes is done which are of our interest. For each organism, gene complement can be analysed to reveal different pathways, e.g. carbon fixation, energy generation, etc. (Tyson et al. 2004), or genes of importance to plants for different functions such as ahpC gene (Lee et al. 2014), hsp90 (Erlejman et al. 2014), albumin, actins, tubulins, cyclophilin, glyceraldehyde-3-phosphate dehydrogenase (G3PDH), hypoxantine phosphoribosyltransferase (HRPT), etc. (Thellin et al. 1999).

Apart from whole microbiome, trends of simultaneous assessment of community structure and function are done nowadays. This is called as 'whole metagenomics'. In whole metagenomics, the structure of the microbial community can be unravelled by both gene-targeted metagenomic (GTM) approach and whole metagenome sequencing (WMS).

In gene-targeted metagenomic approach, a DNA pool is selected and cloned and then subjected to sequencing. GTM can be done by a) sequence-driven screening or b) function-driven screening. Sequence-driven screening is done by using 16S or 18S ribosomal RNA gene sequence as genetic markers to identify genome fragments of specific group of organisms. It is independent of expression of cloned genes in foreign hosts but the primers and DNA probes are designed from conserved regions. Function-driven screening is a direct route to discover gene clusters. The gene clusters are related to metabolic roles in microbial communities (Suenaga 2012). But this suffers from disadvantages like the following: some genomes lack sequence relatives which may lead to missing novel genes, ORF lacks sequence homology to known genes and can be identified as hypothetical protein, complete sequence analysis of insert may target neighbouring genes, etc. (Vieites et al. 2008; Suenaga 2012).

With WMS, the majority of available genome can be retrieved and the functional gene composition of microbial communities can also be accessed. It can also help to find rare prokaryotic and eukaryotic sequence groups. The limitation of PCR biases is also overcome by WMS (Salvetti et al. 2016). Once the reads are sequenced, redundant and low-quality reads are filtered using different methods, e.g. EuDetect and DeConseq (Nakamura et al. 2011; Hess et al. 2011). Then the reads are assembled using either de novo assembly or reference-based assembly. De novo assembly can be achieved by using tools Velvet (Zerbino and Birney 2008) or SOAP (Li et al. 2008) which are based on de Bruijn graphs. If the closely related reference genomes are available for metagenome assembly dataset, then reference-based assembly can be used which can be achieved by using assembly softwares, e.g. Newbler, AMOS or MIRA (Chevreux et al. 1999). After this, the DNA sequences are sorted into groups using tools such as MEGAN (Huson et al. 2007), S-GSOM (Chan et al. 2008), IMG/M (Markowitz et al. 2007), PhymmBL (Brady and Salzberg 2009), PCAHIER (Zheng and Wu 2010), MG-RAST (Glass et al. 2010), CARMA (Krause

Table 12.2 Comparison of NGS technologies

Technique	Advantages	Accuracy	Disadvantages	References
Single-molecule real-time sequencing (SMRT or PacBio)	Longest read length (10 to 15 Kb) Highest consensus accuracy	87%	Equipments are very expensive	Pollard et al. (2018)
Ion torrent	Rapid sequencing speed Low operating cost	99.6%	Difficult to enumerate long repeats	Davies (2010)
Illumina	High sequence yield	99.9%	Requires high concentration of DNA	Kozich et al. (2013)
Nanopore	Longest individual reads Portable	92 to 97%	Lower throughput than other machines	Branton et al. (2010)
Roche 454	Long read size Fast	99.9%	Homopolmer errors Expensive	Luo et al. (2012)
Sequencing by ligation (SOLiD)	Low per base cost	99.9%	Slower than other methods Palindromic sequences issue	Huang et al. (2012)

et al. 2008), SOrt-ITEMS (Monzoorul Haque et al. 2009), MetaCluster (Leung et al. 2011), etc. Then at last, annotation of metagenome is performed by identifying and predicting genes by FragGeneScan (Rho et al. 2010), Metagene (Noguchi et al. 2008), etc. and assigning putative gene functions by using tools such as KEGG (Kanehisa et al. 2004), COG/KOG (Tatusov et al. 2003), PFAM (Finn et al. 2009), etc (Table 12.2).

12.4 Co-relation Between Soil Microbiome and Plant Genotype

Very much like human gut microflora, the plant microbiome system is occupied by diverse microbial community, establishing a strong functional basis to their respective hosts (Berendsen et al. 2012). The plant roots bridge the complex eukaryote and the surrounding soil which is rich in microbial organization. The bacterial community associated with the soil comes in close proximity with the plant roots and modulates plant growth and development by supplying nutrients and providing stress resistance that operates in an interlocked network mode (Vandenkoornhuyse et al. 2015; Berg et al. 2014a, b). There is limited knowledge about how the root exudates construct the specific rhizosphere microbial diversity (Chaparro et al. 2014) and the chemicals released in the exudates acts as the chemotactic signals to influence the microbial community (Badri et al. 2013a).

The association of plant and its microbiome represents the most investigated area of research in the last few years (Berendsen et al. 2012). The plant-microbe interaction is a complex process that involves a vast array of microbes and the different factors influencing this complex ecosystem. The dynamics of microbial rhizosphere communities is shaped by the different factors such as soil type, plant species, plant developmental stage, climate and geographical location. The plant roots are a key determinant of the rhizosphere microbiome, as it is the roots of the plant that interacts with the surrounding soil. In rhizosphere research, early studies showed 'rhizosphere effect' which depicted enhancement of soil microorganisms resulting from biotic and abiotic alterations of the soil with major emphasis on organic exudates from the plant roots within rhizosphere (Raaijmakers et al. 2009). The differences in the microbial communities are explained by differential gene expression even in the related crops grown in same soil, providing an insight for the plant-mediated selection of taxa in the rhizosphere/rhizoplane (Ofek-Lalzar et al. 2014). Plant roots grow in soil which is highly diverse and abundant in microbial communities but only colonizes specific and taxonomically limited root-associated microbiome. It was shown in isogenic mutants of *A. thialiana* that plant's innate immune system also plays a role in selection of bacterial taxa in rhizosphere. It was seen that salicylic acid, a phytohormone, also plays a role in plant defence and to an extent modulates the root colonization by specific bacterial communities (Lebeis et al. 2015). The plant's evolutionary history can also influence root colonization by microbes even when different genotypes of the same plant are grown in the same soil (Manter et al. 2010; Bouffaud et al. 2014). However, in comparison recent studies on 16S rRNA gene amplicon sequencing of *A. thaliana* revealed a weak 'rhizosphere effect'. OTU (operational taxonomic unit) richness shows slight differences in taxonomic composition and community structure in the rhizosphere soil (Bulgarelli et al. 2012; Lundberg et al. 2012).

Rhizosphere microbiome is modulated by plant in a host-dependent way. Each plant species uphold a particular set of rhizosphere microbiome and hence the microbiome modulation is host-dependent (Turner et al. 2013; Ofek-Lalzar et al. 2014) which is clearly a plant host effect and microbial host preference (Badri et al. 2009), demonstrated the participation of ATP-binding cassette (ABC) transporters in regulation of secretion of phytochemicals in the roots thus modulating the production of phenolics and sugars in ABC transporter mutants of *Arabidopsis thaliana* than in the wild type thus resulting in accumulation of more potential microbiome in the rhizosphere. Another study showed that the glucosinolates are the bioactive chemicals that are naturally produced by *Arabidopsis*. In transgenic mutant producing an exogenous form of glucosinolates showed a varied effect on fungal and bacterial composition of rhizosphere, hence establishing the effect of plant genotype on microbiome (Bressan et al. 2009). *Avena strigosa* (oats) is reported to produce triterpenoid saponins known as avenacins, which shows antifungal activity (Maizel et al. 1964). Mutants lacking avenacins demonstrated different culturable composition of root colonizing fungi than the wild type and the mutant variety was seen to be more susceptible to fungal pathogens (Osbourn et al. 1994).

The microbial composition varies not only with the soil source but is influenced by the plant genotype even under controlled greenhouse conditions (Edwards et al. 2015). In addition to soil, seeds that contain genetic blueprint of plants are reservoirs of diverse microbiota. The seed acts as the principal source of microbial inocula in plant. It has been reported that seed carried microorganisms effect process of seed germination and seedling survival (Truyens et al. 2015). The transfer of endophytic bacteria takes place through vertical transmission, from host plant to seed and then to seedlings as reported in case of rice and wheat (Robinson et al. 2016). It is seen that microbial diversity also varies with different developmental stages. The analysis of the microbial community of *Arabidopsis* at the seedling stage was found to be distinct from vegetative, bolting and flowering stages of the plant (Chaparro et al. 2014). In contrast, some studies suggested that the root microbiota are assembled in the early plant life and are independent of the plant developmental stage. There were no significant differences in the structural microbiota of an early flowering *A. alpina* (mutant) compared to the non-flowering wild-type plants at the same age, suggesting the developmental status is not much responsible for the compositional changes of the host plant microbiota (Chaparro et al. 2014; Dombrowski et al. 2017).

Regardless of the complex diversity in natural environment, there is a need to overlook the plant microbial diversity when interpreting the dynamics of the host plant. Genetic manipulation of plants for disease resistance or crop improvement may have unforeseen effects on the rest of the microbial diversity, which might not be physiologically relevant. The role of the microbiome in regard to the plant health, biogeochemical cycles, productivity and crop improvement should be seen as much as the plant itself.

12.5 Microbiome Dynamics vis-a-vis Plant Growth Phases

The microbiome of a plant can be described as the sum of all the microbial associations with plant in various plant compartments. The study of plant microbiome basically involves the study of its rhizosphere and phyllosphere. The rhizosphere of a plant represents the soil-plant interface and the phyllosphere forms the air-plant interface (Berg et al. 2014a, b). In other words, we can say that all the microorganisms associated with the plant in the below-ground parts are included into the rhizosphere where the microbes associated with the above-ground parts of the plant are a member of phyllosphere. Both these zones are of immense importance to the plant because of the diverse microbial richness including bacteria, fungi, archaea and protists. All the microbial activities in these areas directly/indirectly affect the plant because these can be beneficial to the plant or may affect the plant health by acting as plant pathogens. The rhizospheric soils of different plants represent a region of extreme microbial activity which is mainly due to the release of root exudates (Bowen and Rovira 1999). The release of unique cocktail of exudates by different plant species attracts a specific bacterial assemblage. The phyllosphere of a plant is a habitat for a large and complex microbial community. The diversity of microbes associated with the above-ground parts of the plant is of special interest due to the

large and exposed surface area of plant and is often influenced by numerous environmental factors and physio-chemical properties of the plant (Whipps et al. 2008; Rastogi et al. 2013). However, in addition to rhizosphere and phyllosphere, plants can be sub-grouped into even more microenvironments, such as anthosphere (flower), endorhiza (root), spermosphere (seeds) and the carposphere (fruit) (Berg et al. 2014a, b). All these microenvironments are responsible for providing conditions important for microbial life, thereby specifically affecting the host functioning. The plant microbiome is not only specifically affected by the root exudates or the above-ground interactions but the ageing of the plant has been reported to play a major role in this process as well. Many reports have shown that with the changing growth phase of a plant, its microbiome is severely affected.

12.6 Rhizosphere Microbiome

Rhizodeposits, exudates released by the plants in rhizosphere, are known to be important for variations in the diversity of microbial communities (Smalla et al. 2001). These include water-soluble exudates along with complex organic compounds from dead root cells (Bowen and Rovira 1991). These deposits are also important to plant for growth and disease reduction, for example, some of the plant synthesized sugars, i.e. sucrose released by roots in the rhizosphere is then responsible for the production of plant growth-promoting phytohormones. Exudation patterns undergo certain changes in relation to different plant growth stages, e.g. a higher concentration of exudates is seen during the early growth phases which gradually decreases as the plant ages. This change in the plant life cycle and exudation pattern has often been correlated with the type of microbiome diversity as reported by Yang and Crowley (2000).

The release of photosynthates, for example, and the corresponding composition of rhizodeposits in the rhizosphere have been shown to vary throughout the plant's life cycle due to the changes in plant physiology which occur during the course of development (Gransee and Wittenmayer 2000). The carbon allocation in the below-ground parts has been shown to decrease with the increasing age of plant. This spatial and temporal variation of carbon source effects the composition of rhizosphere microbial diversity. Mougel and co-workers in 2006 reported, during reproductive stages in *Medicago truncatula*, that there is a significant decrease in the root/shoot partitioning of carbon. They explained that this decrease occurred as photosynthates were used more in the shoots than in the roots, which resulted in less release of organic compounds in the rhizosphere during reproductive stages over vegetative stages. Considerable carbohydrate changes have also been noticed especially during the flowering stages of most of the plants. During the vegetative phase of plant development there is more release of soluble root exudates, whereas older plants have more organic compounds derived from dead root cells, especially during seed maturation (Eissenstat and Yanai 2002).

In addition to the variations in the exudation pattern based upon plant growth phase, bacterial and fungal diversity differentially use organic compounds. Bacteria

mostly have higher metabolic reactivity compared with fungi. Bacteria use readily available organic compounds, whereas fungi use complex organic compounds for their metabolism as they possess enzymatic activities (De Boer et al. 2005). This could be co-related with the higher bacterial diversity in the rhizosphere during the vegetative phase of plant growth as a result of increased release of soluble root exudates, whereas, during pod maturation, there is decrease in the amount of rhizodeposits and an increase in complex organic compounds which favours the persistence of fungal diversity at that particular phase of plant growth.

Many studies have shown a change in the rhizospheric fungal and bacterial communities based on plant's developmental stages in a wide variety of plants (i.e., *Arabidopsis*, maize, *Medicago*, pea, sugar beet and wheat). Micallef et al. (2009) reported that rhizosphere microbial communities in Arabidopsis varied with plant developmental stages and microbial community diversity was completely different in early stages of plant development as compared to the microbial diversity of the later stages. In another finding, soybean rhizosphere microbial communities were studied for a change in overall pattern with respect to the plant growth phases and it was seen that more complex microbial communities were produced during early reproductive growth stages of the soybean plant as compared to late stages of plant development (Xu et al. 2009). The characterization of the *Arabidopsis thaliana* core microbiome provided a tool to decipher the influence of different plant growth phases on the rhizosphere microbiome (Lundberg et al. 2012). Chaparro and co-workers in 2014 reported that the root exudates composition changes with respect to plant developmental gradient in *Arabidopsis.* During early time points, sugars, sugar alcohol level secretion, amino acid secretion and phenolics concentration were higher and with plant growth the microbiome diversity decreased. Thus metabolites and substrate secretion select particular microbiome at different life cycle stages (Badri et al. 2013; Chaparro et al. 2013). The rhizosphere microbes selected at particular plant growth phase is usually associated with suppress pathogenic microbes by beneficial ones (Mendes et al. 2011), induce systemic resistance against abiotic stress (Selvakumar et al. 2012), help in nutrition uptake (Bolan 1991; Van Der Heijden et al. 2008), increase the plant's innate immunity (Zamioudis and Pieterse 2012), and in overall plant health (Berendsen et al. 2012; Chaparro et al. 2012).

For the better understanding of dynamics of microbial compositions (microbiomes) over the plant life cycle, Edwards and co-workers in 2018 studied root compartments to characterize the root-associated microbiota of *Oryza sativa* over a period of three consecutive growing seasons. They observed that root microbiota was highly dynamic during the early stages of plant growth. There was compositional stabilization of microbiome for the rest of the life cycle and it was observed that microbiota of drought-stressed plants is immature as compared to unstressed plants. Qiao and group in 2017 compared the community structure of the rhizosphere bacteria of two different cotton cultivars of cotton through high-throughput sequencing technology and found the root-associated microbiome varied significantly during different developmental stages (Qiao et al. 2017).

The impact of plant growth phases in deciding the rhizospheric community can be estimated from a study conducted by Dunfield and Germida in 2003, where they

tried to assess the difference in the rhizospheric microbial diversity of field grown genetically modified and wild-type canola. They observed changes in the microbiome structure associated with genetically modified plants but found them to be temporary as this change did not persist into the next field season.

12.7 Phyllosphere Microbiome

Phyllosphere is habitat for microorganisms in the aerial parts of living plants that includes buds, flower, fruits, leaves and stems (Whipps et al. 2008). Though the phyllosphere of most of the plants, unlike rhizosphere, has not been very well studied, but it is important for the plant as it hosts a variety of microbes which play important role in the growth and development of the host plant. For example, disease resistance and plant growth is increased in presence of beneficial microbes. In most of the plants the rhizosphere and phyllosphere communities fail to share common constituents, clearly exhibiting differences in dominance pattern of microbial communities. Microbial communities associated with the above-ground parts of the plant are influenced by both biotic and abiotic factors, along with plant surface topography and chemistry. Among all the above-ground plant parts, leaves are the most preferred habitat for colonization of microbes due to its large surface area globally.

Bacteria are the most dominant species in the phyllosphere when compared to archaea, filamentous fungi and yeast. The bacterial species from the phyllosphere promote plant growth directly as well indirectly. Fungal phyllosphere endophytes may deter herbivores, protect against plant pathogens and increase drought tolerance in the host. There are many reports suggesting that the microbial community associated with the leaves of the plants changes with the ageing of the leaves, for example *Zea mays* L. leaf microbial community structure was studied using molecular and microscopic strategies and it was seen that the diversity changed with plant age (Manching et al. 2017). There are many studies that have reported changing pattern of phyllosphere community in relation to plant age. Osono and Mori in 2005 reported phyllosphere fungi of giant dog wood changes in seasonal and leaf age-dependent manner and also the composition of assemblages of phyllosphere fungi was influenced by phenological patterns of leaf emergence of deciduous trees.

In order to have complete understanding of the plant health and for designing the strategies for the development of plant growth-promoting and disease control bio-formulations, knowledge about the microbiome, both rhizosphere and phyllosphere, is very important as this will open new ways for sustainable agricultural practices and meeting the ever increasing food demand. For the efficient study of microbiome of plant, two major approaches have been utilized: culture-dependent and culture-independent.

An enhanced understanding of the factors that influence beneficial microbial behaviour in the phyllosphere and rhizosphere of the plants is of extreme importance in agriculture to enhance productivity and limit environmental impact while maintaining food safety.

12.8 Suppressive Soil Microbiome

Plants have always been greatly influenced by the soil in which they grow. The soil associated with the below-ground parts of most of the plants can be characterized into two major groups: conducive soils and suppressive soils. Conducive soils or non-suppressive soils are those which provide all the necessary biotic and abiotic conditions required by a pathogen to grow and survive in that habitat whereas suppressive soils prevent soil-borne pathogen establishment and also help in disease suppression (Durán et al. 2017). Both the types of soils differ in their microbial composition as well due to the different physical properties. Young and co-workers in Young et al. 1991 studied three conducive and suppressive soils for various physical and chemical properties and found that suppressive soils were slightly alkaline and conducive soils were slightly acidic. Concentrations of calcium and magnesium were 3–15 times more in suppressive soils compared to conducive.

Suppressive soils, limiting the growth of soil-borne pathogen, have been studied worldwide from the last 60 years (Schlatter et al. 2017). Atkinson in 1892 for the first time reported the suppressive soil for *Fusarium* wilt disease of cotton (Atkinson 1892). Baker and Cook in 1974 described disease-suppressive soils as the soils associated with the plants, which do not allow the pathogens to persist and even if it persists the severity of the disease is very less. Based upon this definition, suppressive soils can be considered as best example of microbe-based plant defence. The plant roots release exudates, attracting the soil microbes and supporting selective microbial community in the rhizosphere. As a result of this, the soil becomes a rich source of beneficial microbes with novel antimicrobial compounds and plant protective traits and this becomes the first line of defence against soil-borne pathogens. These microbes associated with the plant provide protection by competition for nutrients, antibiosis and induction of host resistance (Mazzola 2002). In general two types of suppressiveness are observed: general and specific. When the collective microbial community associated with the plant competes for the available nutrients in that particular niche with the pathogens and suppresses the pathogen growth and development then this is called as general suppression. General suppression is more natural and pre-existing property of soil and this type is usually effective against a broad range of soil-borne pathogens. This type of suppression can be further enhanced by the addition of organic matter in the soils, as this will further support the growth and persistence of plant beneficial microbiome, thereby better plant growth and health but it cannot be transferred from field to field and soil to soil. The other major type of suppression is Specific, and this is attributed to the activity of a specific group of microorganism which inhibits the pathogen progression by interfering in the life cycle of the soil-borne pathogen. Specific suppression is highly effective and is transferable in contrast to general suppression. By mixing small amounts of (1–10% w/w) these specific suppressive soils, we can convert the conducive soils into suppressive soils.

Many research groups have been trying to understand that how indigenous microbiomes are capable of disease reduction in the presence of pathogen, susceptible host and favourable environmental conditions. New molecular biology tools in

Table 12.3 Examples of disease-suppressive soils

Pathogen	Crop	Technique	Abundant microbial taxa	References
Gaeumannomyces graminis var. *tritici*	Wheat	16S - DGGE	*Pseudomonas putida*, *Pseudomonas fluorescens*, *Nocardioides oleivorans, Streptomyces bingchengensis*, *Terrabacter*	Chng et al. (2015)
Fusarium oxysporum	Vanilla	16S - Amplicon	Acidobacteria (groups Gp2, Gp1, Gp3, Gp13), Verrucomicrobia, Actinobacteria (Ktedonobacter), Firmicutes	Xiong et al. (2017)
*Rhizoctonia solani*AG3	Potato	16S - Amplicon	Proteobacteria, Bacteroidetes, Actinobacteria, Acidobacteria	Michelsen et al. (2015)
Heteroderaglycines	Soyabean	ITS - DGGE	*Fusarium* spp., *Cladosporium sphaerospermum*, *Aspergillus versicolor*	Song et al. (2016)
Fusariumoxysporum f. sp. *cubense*	Banana	16S - Amplicon	Acidobacteria (Gp4, Gp5), *Chthomonas, Pseudomonas*, *Tumebacillus*,	Shen et al. (2015)

Source: Gómez Expósito et al. (2017)

combination with the traditional approaches have been used to understand the suppressive soil microbiomes (Cha et al. 2016). Techniques such as metagenome, metatranscriptome, metataxonome in combination with bioinformatics approaches such as metaproteome and metabolome have helped in unravelling the spatial and temporal components of general and suppressive soil microbiomes. By the study of these soils, we can identify microbes which confer disease suppressiveness by making a microbial consortia and by transplanting the microbial communities, soil microbiome engineering (soil amendments) or plant-mediated microbiome engineering (exudation patterns) (Gómez Expósito et al. 2017) (Table 12.3).

12.9 Saffron Microbiome

In the past 10 years, we have taken an initiative to study saffron microbiome and its interactions, from the saffron fields in Kashmir and Kishtwar. Our focus has been mostly on the microbiome associated with the underground parts of saffron such as the rhizosphere and cormosphere. Both cultivation-dependent and cultivation-independent methodologies were used for elucidation of structure and function of the saffron microbial community and study their spatial and temporal dynamics.

Fungal pathogens have been isolated from rotten corms to study the effect of saffron associated bacteria on fungal pathogens causing corm rot diseases in saffron. *Fusarium* corm rot is reportedly the most destructive disease in saffron-producing

areas worldwide. Three fungal pathogens, *Fusarium oxysporum, F. solani* and *Penicillium* sp., have been isolated from saffron fields in Kashmir and identified using molecular phylogeny besides conidial morphology. Out of these three pathogens, *F. oxysporum* R1 shows maximum disease incidence and disease severity is also higher and was found to be closely related to *F. oxysporum f.sp. dianthi* based on ITS sequence phylogeny (Gupta and Vakhlu 2015). Since individual strains of plant pathogenic *F. oxysporum* are host specific despite their wide host range, it is important to characterize the strains. Earlier reports on saffron pathogens lack molecular characterization. Characterization of pathogenic strain infecting saffron would therefore facilitate screening for biocontrol bacteria targeted against the disease.

Plant growth-promoting bacteria (PGPB) have been isolated from field soil, rhizosphere and cormosphere and studied for plant growth-promoting properties, namely, protease production; production of amylase, indole acetic acid, ammonia, catalase, siderophore and cellulase; phosphate solubilization; and antifungal activity. These studies included both *in-vitro* tests and pot trials (Ambardar and Vakhlu 2013; Kour 2014). Bacilli spp. namely *B. thuringiensis* DC1*, B. amyloliquefaciens* DC8 and *B. megaterium* VC3 have been characterized from cormosphere and were found to be present in the corm throughout its life cycle (Kour 2014; Kour et al. 2018). *B. methylotrophicus* and three different strains of *B. aryabhattai* have been characterized from bulk soil. *B. aryabhattai* has also been isolated from rhizosphere (Ambardar and Vakhlu 2013). *B. amyloliquefaciens* strain W2 has been found effective against corm rot caused by *F. oxysporum* R1, using well diffusion and dual culture assays and pot trials. It has been shown to decrease the disease incidence in pot assays from 93% to 40% (Gupta and Vakhlu 2015). The genomic DNA of *B. amyloliquefaciens* W2 has been isolated and sequenced using Ion PGM sequencing platform. The draft genome (3.9 Mb) has 65 contigs (3, 997, 511 bp), 4,163 coding sequences, and an average 46.45% GC content and is available at GenBank. Though comparison of 16S rRNA gene of isolated BamW2 showed 99% similarity with that of *B. amyloliquefaciens* subsp. *plantarum* FZB42, the genome sequence comparison revealed only 48.7% homology between W2 and FZB42 strains (Gupta et al. 2014).

In order to understand the interactions between saffron, its pathogen and native biocontrol agent, transcriptome sequencing-based study with W2 strain as biocontrol agent has been undertaken. Plant gene expression and fungal gene expression in presence and absence of biocontrol agent have been studied and compared to understand the effect of pathogen on healthy corm and infected biocontrol-treated corm. The comparison has revealed differential expression profiles of plant genes as well as fungal genes in both the samples (Unpublished data).

Cultivation-independent approach has unravelled a detailed picture of microbial diversity associated with corms and roots of saffron. Two approaches were used for this study, namely, cloning-dependent and high-throughput sequencing. Cloning-dependent approach included cloning of 16S rRNA gene which was amplified from the metagenomic DNA of the sample. This study revealed that the bacterial composition of bulk soil, rhizosphere and cormosphere of saffron is significantly different ($P < 0.05$) from each other. Briefly, in the flowering stage, rhizosphere and cormosphere of saffron have 22 bacterial genera but none of the genus is common. Bulk soil bacterial community comprises of 13 genera with *Acidobacteria* as the dominant one. In

rhizosphere, 8 different genera were identified and *Pseudomonas* was the most dominant. Cormosphere community, dominated by the genus *Pantoea*, comprised of 6 different bacterial genera. This was the first report on bacterial community structure of saffron plant parts using cultivation-independent 16S metagenomic approach (Ambardar et al. 2014). However, cloning-based sequencing has inherent biases and underestimates community diversity (Ambardar et al. 2014). Therefore, high-throughput sequencing approach is preferred for deep analysis of microbial diversity (Tedersoo et al. 2010). Using this approach for sequencing, 23 bacterial genera were revealed from cormosphere (unpublished work). This number was significantly higher than 6 genera found by cloning-dependent technique, though different life cycle stages under investigation were different (Ambardar et al. 2016a).

Similarly ITS gene high-throughput sequencing was used to catalogue fungal diversity from bulk soil, rhizosphere and cormosphere of saffron. The analysis of 454 pyrosequencing data has suggested niche and growth stage-specific nature of saffron mycobiome. Fungal diversity obtained was different between roots and corms and the dominance pattern in the cormosphere varied among two growth stages. Briefly, during flowering stage, Zygomycota and Basidiomycota were dominant fungal phyla in the rhizosphere and cormosphere respectively. However, in the cormosphere the dominance pattern shifted from Basidiomycota to Zygomycota from flowering to dormant growth stage. On the hand, the bulk soil fungi do not seem to follow this dynamics and was dominated by Ascomycota throughout the study. This was the first report on the fungal community structure and its spatial and temporal dynamics in saffron (Ambardar et al. 2016a, b). To compare microbial diversity of saffron cormosphere from different geographical sites, corm samples from Kashmir and Kishtwar have been further studied to unravel their microbiomes. Subsequently, the technique for studying microbial diversity/microbiome was based on whole metagenome sequencing instead of initial gene-targeted sequencing technology as sequencing is getting cheaper day to day. Preliminary analysis of the microbiome of two sites reveals that the microbial diversity of both Kishtwar and Kashmir cormosphere is different (Unpublished work).

12.10 Microbiome Engineering: Future Aspects and Challenges

Microbiome engineering is alteration of microbial compositions to improve host phenotypes and benefit ecosystems (Foo et al. 2017). It can be a valuable tool to improve agricultural production and increase food security in light of climate change and a growing human population. Manipulation of host microbiome for increased health and productivity is the prime goal of microbiome engineering. The plant beneficial functions are thought to be carried out by a few microbial species by their synergistic effects rather than the whole microbiome. These few species, associated with plants, can be used to benefit susceptible plants against biotic stresses and can also help to increase yield by plant growth promotion. It is therefore essential to elucidate microbiome structure and functions as well as their effect on host's performance (Mueller and Sachs 2015).

Host-mediated artificial selection, microbiome transfer and synthetic microbiomes are the methods that have been used to engineer root-associated microbiome. One of the methods for microbiome transfer for plant disease management is transferring disease-suppressive soils, and this method has been used successfully in potato common scab, sugar beet infection and tobacco black root rot (Foo et al. 2017). Microbial species can be introduced into host by plant multi-generation-host-mediated microbiome selection. Inoculation can be provided to bulk soil, rhizosphere, seeds or seedlings. At the tissue level, inoculation can be done by atomization directly into stems, leaves and flowers, or direct injection into tissues or wounds. Host-mediated microbiome selection is an engineering method that selects microbial communities indirectly through the host. This method leverages host traits that evolved to influence microbiomes and was demonstrated first on *Arabidopsis* (Mueller and Sachs 2015; del Carmen Orozco-Mosqueda et al. 2018). Inoculation into bulk soil and rhizosphere includes introduction of plant growth-promoting (PGP) microbes that may change the structure of plant microbial community. Inoculation of microbial species has also been shown to be effective on seeds and seedlings. Tissue atomization is another technique that has been used to modify seed microbiome (Mitter et al. 2017). Direct inoculation of a PGPB into a plant has been demonstrated to help the bacterium to colonize and survive within the plant. For example, the biocontrol agent and PGPB *Arthrobacter agilis* UMCV2 is reported to survive, after being injected to the stem of *Medicago truncatula* plants (Aviles-Garcia et al. 2016).

Microbiome engineering has an advantage over single gene transfer, since, transfer of one or more microbial species leads to a transfer of greater concentration of genetic material and thus provides greater advantage to plants compared to single gene transfer. For example, the multiple direct and indirect growth-promoting activities of PGPB such as *Pseudomonas fluorescens* UM270 or *Arthrobacter agilis* UMCV2 can be more beneficial than the single gene transfer for *cry* gene from *Bacillus thuringiensis* (del Carmen Orozco-Mosqueda et al. 2018).

Commercialization of microbial bioformulations, for enhancing farm productivity, has gained momentum in recent years with the help of start-up companies (Indigo Ag, Chr-Hansen, NewLeafSymbiotics) as well as multinational companies (Bayer Ltd, Nufarm, Monsanto BioAg). Microbial products and bio-pesticides are expected to grow in the global market to an estimated $6.4 billion by 2022 (Singh et al. 2018). However, there are significant challenges along the road ranging from technical to social. Additional studies to understand the interactions and impact of microbial species on the plant's core microbiome are essential. Along with that, more than 95% of microbes are non-cultivable and hence not characterized yet, constraining the ability to harness their potential for agricultural improvements. Performance in field is another challenge, as the reports have shown mixed results. The impact of environmental factors as well as plant's genotype on microbiome diversity poses a challenge for microbiome engineering particularly for phyllosphere. It is therefore important to study the plant-microbiome interaction taking into consideration effects on both plant and microbial participants. Re-isolation of introduced microbial species is another important aspect to ensure its endophytic

capacity. Effect on the core microbiome over several generations and long-term persistence of the engineered microbiome are some studies that should be taken into consideration. Another area of focus for future research is the understanding of how plant's genetic pathways shape/influence microbiome. There is only limited knowledge of how root exudates select microbiome for plant's advantage. Together, the omics studies can help elucidate this plant-microbiome relationship in depth. This knowledge can be further exploited to improve crop production and reduce dependence on chemical fertilizers by microbiome engineering (Bakker et al. 2018).

Climate change is a major challenge and a threat to food security in the present scenario. This threat to food security can also be managed using microbiome engineering with the integration of systemic biology, ecology and evolutionary biology. The impacts of climate change on soil microbial community composition and metabolic diversity can be predicted by ecological studies and can be complimented by evolutionary studies that will predict stability of synthetic microbial communities and microbial mutualisms over the time. Further field experiments can be conducted for evaluation of external factors on microbial community composition and synergistic benefits of inoculations (Hamilton et al. 2016). A stable microbiome which can improve productivity and stress tolerance under diverse environmental conditions and crop stages is highly desirable.

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Part II

Diversity of Specific Microbial Groups

13 Diversity of Lichen Photobionts: Their Coevolution and Bioprospecting Potential

Khem Chand Saini, Sanjeeva Nayaka, and Felix Bast

Abstract

Lichens are the symbiotic association of green algae (phycobiont) or blue-green algae (cyanobiont) with fungus (mycobiont). Lichen-forming fungi consist of about 20,000 species, whereas the known photobionts are only about 156 species from 56 genera. A confounding reason for this disparity in the species richness is that most of the lichenologists are mycologists and their focus is on the mycobionts rather than photobionts. Therefore, mycobionts are comparatively well-characterized while the real diversity of photobionts remain elusive. Diversity and phylogeny of major photobiont lineages described till date are comprehensively covered in this systematic review, along with the data on ecology, patterns of phylogeography, and evolution. Current understanding of photobionts described from the Indian subcontinent is summarized revealing significant knowledge gaps in this field. Given that photobionts have relatively simple morphology and morphological plasticity, the relevance of DNA sequence-based molecular systematics for photobiont characterization is highlighted, and other challenges in photobiont research are discussed.

Keywords

Symbionts · Lichenized fungi · Molecular systematics · Mycobionts · *Nostoc* · *Trebouxia*

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13.1 Introduction

Lichens are the symbiotic association of green algae (phycobiont) or blue-green algae (cyanobiont) with fungus (mycobiont). In some cases, both green and blue-green algae associate to form lichens. They are often referred as best example of mutualistic relationships (Fig. 13.1). The mycobiont forms a physical scaffold that encloses and supports the growth of photobionts, while photobionts, as they can photosynthesize are the sole source of carbohydrate for the fungal partner (Fig. 13.2) (Galloway 1992; Hawksworth 1988b; Honegger 1991).

The Swiss botanist Schwendener (1867) was the first to demonstrate that the tiny green bodies in lichen thalli, that he referred as gonidia, were cyanobacteria or green algae rather than fungal mycelia. He claimed that lichens are composite organisms encompassing two separate species, viz. fungi and algae, which brought a paradigm shift in lichenology. Nature of association between fungus and algae

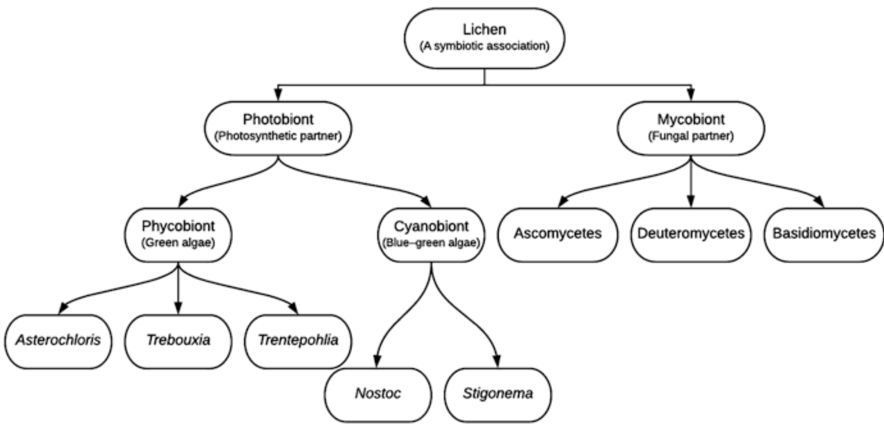

Fig. 13.1 Graphical representation of lichen association

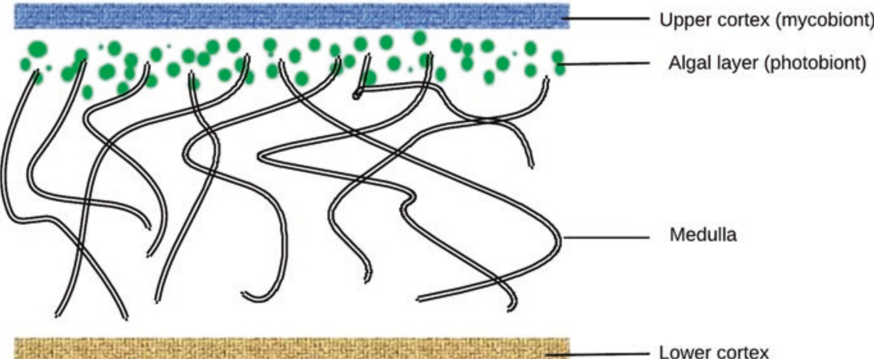

Fig. 13.2 Vertical cross section of lichen thallus

were at first thought by Schwendener as parasitism (Schwendener 1867), but several later scientists proposed alternate association scenarios including mutualism/consortium (Reinke 1872), symbiosis (unlike organisms living together, De Bary 1879), endo-saprophytism (Elenkin 1902), helotism (master and slave relationship, Nienburg 1917), and controlled/balanced parasitism (Ahmadjian and Jacobs 1983). The current scientific consensus is that the relationship is a form of parasitism as initially suggested by Schwendener, as the algal partner can very well live freely without the fungal partner, while the other way round is impossible. After 150 years of Schwendener's discovery, Spribille et al. (2016) discovered an unexpected third partner in lichens, the basidiomycete yeasts embedded in the cortex of the ascomycete macrolichens. Basidiomycete yeasts are thought to be producing chemicals that help lichens ward off predators and repel microbes (Spribille et al. 2016).

Photobiont in the lichen associations were ignored by lichenologists for long time due to several reasons. First, lichenology is traditionally considered as a subdiscipline of mycology or botany, with mycologists focusing solely on mycobionts of lichens. Second, there is an acute scarcity of trained phycologists competent enough to study the microalgae from its simple, often 'plastic', morphological features. Third, fungal partners were thought to be the dominant component of lichen thallus, as about 90% thallus is made of mycobionts that provide shape, color, and structure to the lichen. Therefore, lichens were taxonomically treated as 'lichenized fungi' by many lichenologists (Nayaka 2014). However, as already explained, this logic is flawed, because the fungal partner has no autonomy without its algal partner. As algal partner is the limiting component of lichens, it is reasonable to infer that the most important component of lichens is the algal partner, although it is being overlooked for the last two centuries. Group of lichen-forming fungi consists of about 20,000 species worldwide (Kirk et al. 2008). Of all known lichenized fungi, 98% are Ascomycetes, 1.6% Deuteromycetes, and 0.4% Basidiomycetes (Nash III 2008). Whereas the number of photobionts is only about 156 species from 56 genera (Anna et al. 2013). Most photobionts belong to green algae (Chlorophyta-116 species, 73.9% of all photobiont diversity) or cyanobacteria (Cyanoprokaryota-35 species, 22.3%). Because of its algal component, the composite organism acquires the photoautotrophic ability, which is crucial for its survival at oligotrophic locations and during primary successions. Such association and its further coevolution allowed lichens to function as pioneer organisms in habitats devoid of soil and organic substances – some of the most unfavorable habitats of the world (Anna et al. 2013).

Three genera (*Trebouxia*, *Trentepohlia*, and *Nostoc*) are the most frequent photobionts in lichens. The genera *Trebouxia* and *Trentepohlia* are of eukaryotic in nature and belong to the green algae, while the genus *Nostoc* belongs to the oxygenic photosynthetic bacteria (cyanobacteria) (Büdel et al. 2009). Among the cyanobacteria-containing lichen species (sometimes referred as "cyanolichens"), 10% are bipartite (having cyanobacteria as the only photosynthetic partner), and 3–4% are tripartite (having two photosynthetic partners, viz., green algae and cyanobacteria) (Rai et al. 2002). In tripartite cyanolichens, cyanobacteria are restricted to an individual gall-like external or internal structure called cephalodia, in which

the fungal partner creates microaerobic conditions to facilitate cyanobacterial nitrogen fixation (Honegger 2001; Rikkinen 2007).

Lichenized fungi exhibit a small number of taxonomically useful characters (i.e., homologous traits), while the vast majority are homoplasious, therefore, of little practical utility. Sometimes, the fine delineation of morphological characters between homologous and homoplasiousis practically difficult within and between taxonomical groups (Lumbsch and Huhndorf 2010; Printzen 2010; Stenroos et al. 2002). Due to the scarcity of taxonomically relevant homologous (and more specifically, orthologous) morphological features, molecular data have, therefore, gained great momentum in lichen systematics. Molecular data have helped in identifying the phylogenetic position of lichen-forming fungi and associated photobionts in a number of studies worldwide (Gargas et al. 1995; Schoch et al. 2009a, b). The advent of molecular systematics had a major revolution in the classification of lichens and its individual algal and fungal partners. DNA sequence data enable the researchers to trace the evolutionary status of these organisms and their associated lineages (Lewis et al. 1992). Currently, DNA sequence-based information contributes a significant role in species identification and inventorying (DNA barcoding), biodiversity characterization and species discovery (DNA taxonomy) and patterns of species dispersion through geography (phylogeography). Phylogenetic analyses of lichens and photobionts are mostly carried out by nuclear SSU, LSU ribosomal DNA (rDNA), and ITS sequences. DNA sequence-based barcodes provide a reliable, cost-effective, and accessible solution to the existing problem of species identification and characterization. Reports have suggested that several of these barcodes have faster rates of molecular evolution and can be used for the intra-specific variation.

13.2 Characteristics of Lichens

The word 'Lichen' (Greek, leichen) was first used by Theophrastus in about 300 BC to describe outgrowths from the bark of olive trees. Lichens are a known bio-indicator of air pollution, and they also function as pioneer species in ecological successions (Ahmadjian 1993b; Conti and Cecchetti 2001; Lücking 1998). Lichens have surprisingly high ecological resilience and adaptability; various types of lichens are found in hot and dry deserts, barren rocky cliffs, and even in Antarctica. The ability to quickly absorb and retain moisture from many sources makes it possible for lichens to grow in extreme environments like deserts, within or on the frozen substrate in the Polar Regions, and on exposed substratum like leaves and barks of the trees, and rocks. Lichens are also found on the marbles of the ancient monuments and old buildings (Moore et al. 2011).

Lichens exhibit a broad range of habitat differentiation. Although a shift of substratum is seen in lichens due to climate change and rapidly changing land-use patterns, lichen genera mostly prefer a particular type of substratum (Tretiach and Brown 1995). The form of lichen vegetation depends mainly upon shape, structure, water relations, and the chemistry of the substrates. On the basis of substrates, the

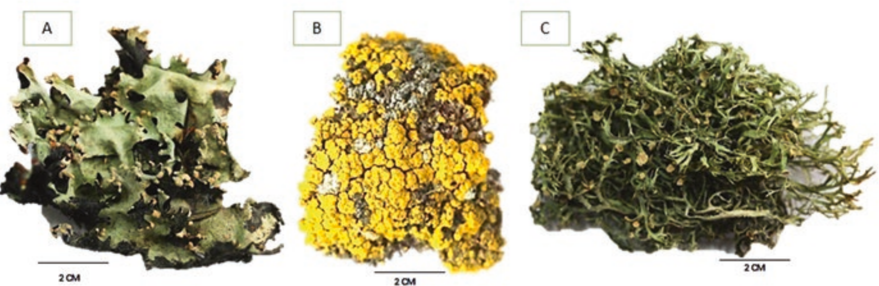

Fig. 13.3 Different growth forms of lichens, (**a**) Foliose lichen (*Parmotrema*), (**b**) Crustose lichen (*Candeleriella*), and (**c**) Fruticose lichen (*Ramalina*)

lichens can be classified into habitat subsets—saxicolous (inhabiting rocks and stones), corticolous (growing on tree barks), terricolous (soil inhabiting), ramicolous (growing on twigs), muscicolous (growing over mosses), and omnicolous (inhabiting various substrates and manmade structures). Among these habitat subsets, corticolous and terricolous lichens are excellent indicators of ecosystem quality (Will-Wolf et al. 2002). On the basis of thallus size, structure, and morphology, lichens belong to three growth forms – crustose (markedly two dimensional and firmly attached to the substrate by their entire lower surfaces), foliose (grow three dimensional in a sheet-like form, not attached by their entire lower surfaces to their substrates, some are just centrally attached) and fruticose (erect or pendulous and markedly three-dimensional) (Fig. 13.3). Crustose and foliose lichens together are classified under 'Microlichen' group, while fruticose lichens under 'Macrolichen' group (Nayaka 2014).

The unique physiological (i.e., poikilohydric metabolism, secondary metabolite production, production of antifreeze compounds, and UV masking agents) and anatomical (absence of waxy cuticle, the lack of root, and absorption of water and nutrients passively from the environment) peculiarities make lichens some of the most tolerant as well as sensitive organisms on the planet. Constraints and vulnerabilities of lichen anatomy and physiology allow them to inhabit all sorts of habitats in major terrestrial biomes of Earth, utilizing all available substrata, i.e., soil, rocks, barks, ice and human-made surfaces like plasters, concretes, and so on (Galloway 1992).

13.3 Characteristics of Photobionts

Morphological, genetic and physiological differences between photobionts have been reported in many studies (Ahmadjian 1993a; Beck et al. 1998; Brickley 2017; Bubrick 1988; Casano et al. 2011; Friedl and Büdel 2008; Lücking et al. 2009; Peksa and Skaloud 2008; Skaloud and Peksa 2010; Thüs et al. 2011). Photobionts in situ and in vitro have very different morphology and assessment of photobiont morphology in vitro is not reliable for lichen taxonomy. Members of green algal

Table 13.1 Common genera of photobionts identified from lichens (Ahmadjian 1993a; Honegger 2009; Thus et al. 2011; Sanders et al. 2016; Benavent et al. 2017)

Sr. No.	Genus	Sr. No.	Genus	Sr. No.	Genus
1.	*Anacystis*	16.	*Elliptochloris*	31.	*Prasiola*
2.	***Asterochloris***	17.	*Gloeocapsa*	32.	*Pseudochlorella*
3.	*Botrydiopsis*	18.	*Gloeocystis*	33.	*Rhizonema*
4.	*Calothrix*	19.	*Heterococcus*	34.	***Scytonema***
5.	*Cephaleuros*	20.	*Heveochlorella*	35.	*Stichococcus*
6.	*Chlorella*	21.	*Hyalococcus*	36.	*Stigonema*
7.	*Chlorosarcina*	22.	*Hyella*	37.	*Tolypothrix*
8	*Chroococcum*	23.	*Leptosira*	38.	***Trebouxia***
9.	*Chroococcidiopsis*	24.	*Microcystis*	39.	***Trentepohlia***
10.	*Coccobotrys*	25.	*Myrmecia*	40.	*Trochiscia*
11.	*Coccomyxa*	26.	***Nostoc***	41.	*Vulcanochloris*
12.	*Dichothrix*	27.	*Nannochloris*		
13.	*Dictyochloropsis*	28.	*Petroderma*		
14.	*Dilabifilum*	29.	*Phycopeltis*		
15.	*Diplosphaera*	30.	*Pleurococcus*		

aThe most common photobiont genera are shown in bold

class Trebouxiophyceae are the photobionts of more than 60% of lichen-forming fungi (Ahmadjian, 1982; Honegger 2009). Common genera of photobionts reported from various lichens till date are presented in Table 13.1.

The most common photobiont genus, *Trebouxia*, is present in approximately 20% of all lichen species (DePriest 2004); within *Trebouxia*, ca24 species have been formally described (Leavitt et al. 2015). *Trebouxia* isolates can now be identified to species level with some degree of confidence. Friedl et al. (1989) used chloroplast and pyrenoid characteristics to differentiate species of *Trebouxia.* Since these characters are stable in situ as well as in culture, taxonomists can now more easily identify specific photobionts. When species of *Trebouxia* that are presently known are grouped according to the lichen from which they were isolated, no consistent patterns can be seen. Representatives from several cyanobacterial genera (*Chroococcidiopsis*, *Gloeocapsa*, *Scytonema*, and *Stigonema*) are known to associate with different lichens, but the most common cyanobacterial genus in lichen symbiosis is *Nostoc* (Rikkinen et al. 2002). The genus *Nostoc* includes filamentous, non-branching cyanobacteria that produce heterocysts – cells specialized in nitrogen fixation, and hormogonia – the motile filaments often involved in initiating the symbiotic associations (Duggan et al. 2013). Lichen lectins play an essential role in the symbiotic interaction between a fungus (mycobiont) and an alga (photobiont). Lectins are proteins or glycoproteins which bind reversibly to carbohydrates that are present on cellular surfaces and facilitate cellular recognition processes in a range of biological interactions (Singh and Walia 2014).

Peksa and Skaloud (2008) observed a remarkable reduction in cell size found in mostly eukaryotic photobionts. Reproductive strategies of algae are generally modified, and at the subcellular level, modifications of structure, size, as well as the

number and distribution of some organelles and cellular structures were detected (e.g., chloroplast, pyrenoid, thylakoid, dictyosomes, etc.). There were attempts on employing 3D live cell imaging to examine changes in the chloroplast structure of *Asterochloris* photobionts (Peksa and Skaloud 2008). Brickley (2017) developed a potential mitochondrial stain MTO (Mitotracker orange) for labeling the chondriome (the total mitochondrial content of a cell) of *Trebouxia* photobionts. MTO stain was used for both 2D and 3D imaging of mitochondrial morphology of photobionts (Brickley 2017).

13.4 Photobionts Reported from India

India is well-known for its rich heritage of biological diversity, in part to its geological and climatological conditions. Several species of lichens are documented from India and many more are yet to be discovered. However, information on photobionts from India is scanty. Only one report has been reported from India on molecular systematic studies of photobionts; the study states that *Asterochloris* sp. of photobionts is associated with *Cladonia* sp. of lichen (Řídká et al. 2014). In another investigation, the diversity of the photobionts of soil crust lichens was based on morphology (Anna et al. 2013).

13.5 Photobionts: A Key Deciding Factor in Ecological Preferences of Lichens

Photobionts play a significant role in determining the lichen morphology, physiology, and habitat of growth. Lichens with green algae can colonize any environment as they can activate their physiology even using water vapors, whereas cyanolichens are restricted to habitats with the dominance of liquid water. Cyanolichens are desiccation-tolerant organisms, which favor humid and shady environments, whereas chlorolichens tolerate dry and high-light environments (Kosugi et al. 2014). Peksa and Skaloud (2011) stated that photobionts from particular algal clades were found to be associated with taxonomically different, but ecologically similar lichens. Two photobiont taxa were differentiated based on their substrate and climatic preferences (Rain and sun exposure). Photobionts exhibit clear preferences for environmental factors. These algal preferences may limit the ecological niches available to lichens and lead to the existence of specific lichen group (Peksa and Skaloud 2011). Henskens et al. (2012) observed that overall thallus appearance does not change whether one or two photobionts are present in the thallus of cyanolichens. Dal Grande et al. (2012) studied vertical and horizontal transmission of photobiont within populations of lichen symbiosis, which plays an important role inshaping the symbiotic association. Buckley et al. (2014) stated that both evolutionary and ecological processes generate diversity in symbiotic association. Williams et al. (2017) suggested that photobiont switching occurs on an evolutionary scale and there is little evidence that suggests an environmentally induced response. Vančurová et al.

(2018) hypothesized lichens to be flexible in the use of the most adapted photobiont for the environment. Singh et al. (2017) hypothesized that macroclimate might influence symbiont association and diversity patterns in lichen symbiosis.

13.6 Molecular Studies of Photobionts

Molecular phylogenies have encompassed almost every branch of biology including lichens. Numerous phylogenetic methods and software packages are now available (Bast 2013). Classical taxonomists make increasing use of molecular data because traditional lichen taxonomy that is based only on morphological synapomorphies is riddled with problems that only independent data from molecular analyses are likely to solve. The one obvious issue that is relatively easy to explain with molecular data concerns the systematic placement of obligately sterile lichens (Printzen 2010). Nuclear rDNA regions such as ITS, nuLSU, and nuSSU; mitochondrial rDNA mtSSU, mtLSU are the most widely used markers to resolve the relationships in lichens (Crespo et al. 2010; Ertz and Tehler 2011; James et al. 2006; Lutzoni et al. 2004; Miadlikowska et al. 2014; Plata et al. 2012; Schoch et al. 2009a, b; Spatafora et al. 2006). Among these, ribosomal ITS seems by far as the primary tool for molecular systematic studies at the species level. DePriest and Been (1992) were the first to publish the ITS1 sequences from lichen symbionts. Within the last 20 years, lichen photobionts have attracted increasing interest, which has led to the unraveling of their diversity and evolutionary relationships using microscopy, ultrastructure, and molecular techniques (Büdel 1992; Friedl 1987; Melkonian and Peveling 1988; Tschermak-Woess 1988). Due to extreme phenotypic plasticity and simple morphology, characterization of photobiont diversity is not reliable based on only morphological characteristics. Molecular systematics based on DNA sequence barcodes are now routinely used for photobiont characterization, as thoroughly reviewed by DePriest (2004) and Rikkinen (2013). Paul et al. (2018) used high-throughput sequencing for inferring photobiont diversity in lichens (Paul et al. 2018). The ITS1–5.8S-ITS2 rDNA region is generally amplified using universal primer ITS4–3′ (5′-TCCTCCGCTTATTGATATGC- 3′; (White et al. 1990) and the algal-specific primer nr-SSU-1780-5′ (5′-CTGCGGAAGGATCATTGATTC-3′; Piercey-Normore and DePriest (2001) widely used DNA amplification for photobionts. The algal-specific ITS primers ITS 1 T (GGA AGG ATE ATT GAA TCT ATE GT), ITS2T (TTC GET GCG TTC TTC ATE GTT), ITS3T (AAC GAT GAA GAA CGC AGE GAA), and ITS4T (GGT TCG CTC GCC GET ACT A) are based on nuclear ribosomal sequences from the Trebouxiophyceae, including species of *Trebouxia* and *Asterochloris* (Bhattacharya et al. 1996; Friedl and Rokitta 1997; Kroken and Taylor 2000). 16S rRNA genes from cyanobiont are widely amplified by using CYA106F (CGG ACG GGT GAG TAA CGC GTG A), CYA359F (GGG GAA TYT TCC GCA ATG GG), CYA781R (GA CTAC WGGGG TATCTAA TCCCWTT), L27F (AG AGT TTGA TC MTGGCTCAG), 355R (ACTCCTACGGGAGGCAGC) primers (Nübel et al. 1997; Lücking et al. 2009).

In the green algal genus *Trebouxia*, the recently demonstrated congruence of morphological and ultrastructural characters with rDNA sequence data resulted in a revised systematic concept of the genus (Friedl and Rokitta 1997). However, the question whether photobionts are important markers for the evolution and systematics of lichens has been controversial (Ahmadjian 1993a). The diversity of lichen photobionts has been widely studied using molecular markers such as the internal transcribed spacer region (ITS) rDNA sequences. In the study by Beck (1999), the isolated photobionts were cultivated and identified by light microscopy. They also complimented their species delineation with DNA barcoding based on ITS-sequence analyses. The examination was restricted to a defined community of lichens *Physcia adscendens*, *Phaeophyscia orbicularis*, and *Xanthoria parietina. Trebouxia arboricola* and *T. jamesii* photobionts have been reported from these lichen species.

According to Hale (1990), most lichenologists believe that photobionts no longer should have taxonomic status in classifying lichens. As stated earlier, most lichenologists are also mycologists, and this conclusion appears to be highly biased. Jørgensen (1996) believes that taxa based only on photobiont differences are valid. Thus, an example from Jørgensen (1996); *Ionapsis*, with a *Trentepohlia* photobiont, can be distinguished from *Aspicillia*, with a *Trebouxia* photobiont, solely by the photobionts. Species delimitation was used for the molecular delineation of phycobiont and mycobiont species. Species delimitation is the process of determining how individuals and populations fit into natural groups (Leliaert et al. 2014; Sadowska-Deś et al. 2014).

13.7 Host Specificity of Photobionts with Mycobionts

Host specificity of the photobionts is determined with lichenized fungi. One algal genus or species can occur in many different, unrelated lichens. In some cases, there is a good evidence that a lichen species can "partner" with one species in a particular geographic region and with a different species partner in another. Selectivity and specificity are related to process and pattern. In the selection process, a symbiont identifies and associates with the most favorable partner. In the specificity pattern, a symbiont associated with a particular partner is due to strong selectivity or strict vertical transmission (DePriest 2004).

During the last 20 years, molecular phylogenetic studies dramatically changed our views regarding the coevolution of lichen partners. Supposed cospeciation and parallel cladogenesis of mycobionts and photobionts have been generally rejected (Piercey-Normore and DePriest 2001; Taylor et al. 2000) and replaced with the domestication model, in which the fungal partner could select the best available photobiont (DePriest 2004). In general, the mycobionts can cooperate with several algal species and switch them (Muggia et al. 2008; Nelsen and Gargas 2009; Nyati et al. 2007; Piercey-Normore 2006; Wornik and Grube 2010). In the same way, several mycobionts can share a single algal partner (Doering and Piercey-Normore 2009; Rikkinen et al. 2002). Moreover, lichen algae and cyanobacteria could exhibit

Table 13.2 Major photobiont lineages and their associated lichens

Sr. no.	Photobiont genera	Lichens	References
1	*Asterochloris*	*Cladonia* spp., *Diploschistes* spp., *Lepraria* spp., and *Stereocaulon* spp.	Moya et al. (2015), Nelsen and Gargas (2008), Park et al. (2015), Peksa and Škaloud (2011), Řídká et al. (2014) and Skaloud and Peksa (2010)
2	*Trebouxia*	*Evernia mesomorpha* *Melanelixia* spp., *Melanohalea* spp., *Montanelia* spp., *Parmelina* spp., *Parmotrema* spp., *Punctelia* spp., *Xanthoparmelia* spp., *Letharia* spp., *Oropogon* spp., *Cetraria* spp., and *Xanthoparmelia* spp. *Ramalina farinacea* *Rhizocarpon geographicum* and *Circinaria gyrosa* *Xanthoria*, *Xanthomendoza*, and *Teloschistes* spp. *X. parietina*, and *A. ciliaris*	Piercey-Normore (2006), Leavitt et al. (2015), Casano et al. (2011), Sánchez et al. (2014), Nyati et al. (2014) and Grande et al. (2014)
3	*Trentepohlia*	*Acanthotrema* spp., *Astrothelium* spp., *Coenogonium* spp., *Cryptothelium* spp., *Cryptothecia* spp., *Graphis* spp., *Laurera* spp., *Racodium* spp., *Thalloloma* spp., *Thelotrema* spp., and *Trypethelium* spp.	Nelsen et al. (2011)
4	*Nostoc*	*Collema* spp., *Lobariaspp., Nephroma* spp., *Parmeliella* spp., *Pannariaspp., Peltigera* spp.,	Elvebakk et al. 2008, Fedrowitz et al. 2011, Myllys et al. (2007), O'Brien et al. (2005), Oksanen et al. (2002) and Rikkinen et al. (2002)
5	*Scytonema*	*Dictyonema*, *Acantholichen*, and *Coccocarpia, Polychidium*	Lücking et al. (2009), Muggia et al. (2011) and Sassaki et al. (2005)

their environmental requirements, which seem to be independent of particular mycobionts.

Meanwhile, molecular approaches have been used to resolve the relationships among photobionts (Beck 1999; Friedl and Rokitta 1997) and to address the issues of symbiont selectivity using a molecular phylogenetic framework (Table 13.2).

13.8 Coevolution with Mycobionts

Coevolution is the reciprocal evolutionary change in interacting species driven by natural selection. It is one of the most important genetic and ecological processes organizing the biodiversity of the Earth (Thompson 2005). Ehrlich and Raven

(1964) were the first to focus on coevolution in their classic paper on the interactions of butterflies and plants (Ehrlich and Raven 1964). Lichens are a perfect example of coevolution. It is because of their unique features and highly integrated nature (Ahmadjian 1987). Lichens are morphologically, physiologically, and chemically different from their component parts (i.e., either of the symbionts separately cultured); they are the products of symbiotic interactions. In most of the lichens, coevolution seems to be highly advanced that the mycobionts can no longer live independently. For example, Ahmadjian reported that the evolution of the fungal partner of lichen with the photobionts had been closely linked, resulting in reciprocal genetic changes in the symbionts (Ahmadjian 1987, 1988). The absence of free-living populations of lichenized fungi shows that lichen symbionts have experienced long periods of coevolution. Hawksworth (1988a) studied the coevolution of fungi with green algae and cyanobacteria in lichen symbioses. Lutzoni and Vilgalys (1995) proposed the genus *Omphalina* and its associated photobionts *Coccomyxa* as a model system for coevolutionary studies on the mutualistic association of mycobionts and photobionts (Lutzoni and Vilgalys 1995).

Many scientists didn't consider coevolution in lichens. Barrett (1983) discussed that there was little evidence that would unequivocally demonstrate that lichen symbionts have coevolved a mutualistic association. Barrett might be correct in assuming that lichens are not mutualistic associations, but according to Ahmadjian (1987), Barrett was incorrect in presuming that coevolution between the symbionts has not occurred. Piercey-Normore and DePriest (2001) studied long-term symbioses of algae and fungi. Such types of associations are hypothesized to have experienced long periods of symbiotic interdependence and coevolution. They tested two crucial aspects of coevolution, cospeciation and parallel cladogenesis. They observed that symbiont phylogenies rejected parallel cladogenesis and minimized cospeciation and concluded that switching of highly selective algal genotypes frequently occurs among these symbiotic lichen associations (Piercey-Normore and DePriest 2001). DePriest (2004) suggested that coevolution of lichen symbionts can be tested only with indirect measures, i.e., specificity and selectivity of symbionts through demographic and taxonomic approaches and cospeciation and parallel cladogenesis through phylogenetic approaches (DePriest 2004). Hill (2009) proposed that photobionts, as compared to mycobionts, have limited capacity to evolve adaptations to lichenization, so he concluded that the symbionts in lichens do not coevolve. This is because lichens do not have a sequential selection of photobiont cells from one lichen into another required for Darwinian natural selection, and there is no sexual reproduction of photobiont in the thallus (Hill 2009). Tunjić and Korač (2013) reported a high level of horizontal transfers shaping the symbiotic association between phycobionts and mycobionts (Tunjić and Korač 2013).

13.9 Future Perspectives and Conclusion

Mycobionts appear to be strongly selective concerning their photobionts, i.e., only a specific photobiont genus constitutes the appropriate symbiotic partners. Therefore, a better knowledge of photobionts can provide new and valuable information for lichen systematics. The association can better be termed parasitism rather than mutualism, as most of the photobionts are also found living independent of mycobionts, while vice versa is not possible. The current scientific consensus is that mycobionts domesticate the photobionts, and terming this domestication as a mutualistic association is fallacious. Lichen systematics had been traditionally dominated by mycologists, which we believe are the root cause for the biased perception and for the skewed manner in which lichens are classified (as of now, photobionts are not taken into consideration in lichen taxonomy). A call to take a balanced and neutral approach in lichen taxonomy giving equal importance to its photobionts is emphasized herewith. Virtually no research has been focused on bioactivity assays or other potential bioprospecting attempts of photobionts, which needs attention. In lichenized algae and fungi, the advent of DNA sequence-based approaches and advances in molecular systematics have revolutionized our understanding of molecular systematics and evolutionary relationships. Molecular data have helped in identifying the phylogenetic position of lichenized algae and fungi as well as the coevolution among these symbionts. Above all, there is an urgent need for further detailed and focused studies on lichen photobionts with extensive taxa sampling, as the field remained elusive and overlooked for a long time.

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Diversity in Xylan-degrading Prokaryotes and Xylanolytic Enzymes and Their Bioprospects

14

Digvijay Verma, Ravi Kumar, and Tulasi Satyanarayana

Abstract

Heterogenous nature of xylan leads to the multiplicity in xylanolytic enzymes for its complete degradation, where β-1,4-endoxylanases and β-xylosidases play a key role due to their direct action on glycosidic linkages of xylan backbone. α-Glucuronidase (EC 3.2.1.139), α-L-arabinofuranosidase (EC 3.2.1.99), acetyl xylan esterase (EC 3.1.1.6), and lytic polysaccharide monooxygenase (LPMO) are the other prominent players in the xylanolytic enzyme system. Xylan-degrading enzymes are produced by a large number of bacteria and a few archaea. These share a major chunk of industrially relevant enzymes because of their immense potential and widespread applications in several industries that include food/feed, paper, textile, oil extraction, bioethanol, prebiotics, and de-inking waste paper. Microorganisms are a rich source of xylanolytic enzymes. Xylanases produced by bacteria and archaea are functional in broader pH (5.0–10.0) and temperature (30–100 °C) ranges. Easiness in cultivation, rapid growth rates, and well-established methods of DNA manipulations make bacteria preferred microbes over others for xylanase production. This chapter deals with the diversity of bacteria and archaea which produce a variety of xylan-hydrolyzing enzymes and their widespread applications.

Keywords

Bacteria · Archaea · Metagenomics · Xylanases · Xylooligosaccharides · Pulp bleaching · Dye · Decolorization · Bioethanol

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14.1 Introduction

Plants synthesize approximately 10–50 × 10^{12} tonnes of lignocellulosic material annually, which constitute almost half of the polysaccharides present on the earth (Ahmed et al. 2011). Homopolysaccharides of xylan are solely composed of xylosyl residues and commonly present in esparto grass (Chanda et al. 1950) and tobacco stalks (Eda et al. 1976). Hetero- or homo-xylans are the rich source of pentose sugar xylose along with other sugars like arabinose, galactose, glucose, and mannose. Arabinoxylan comprises O-acetyl-(4-O-methylglucurono)xylan, where arabinose residues of barley straw are esterified with p-coumaric acid and ferulic acid at every 31st and 15th position, respectively (Timell 1964; Mueller-Harvey et al. 1986). Xylan polysaccharides form covalent and non-covalent linkages with cellulosic fibers and lignins to maintain the structural integrity of the cell wall (Hori and Elbein 1985; Coughlan and Hazlewood 1993). These bonds can be broken either by mechanical methods or using hydrolytic enzymes. The role of enzymes in the breakdown of xylan was first observed by Hopper-Seyler over 100 years ago (Bastawde 1992). Due to the heterogeneity in xylan composition, a multiplicity in xylanolytic enzymes exists which synergistically act on xylan to release lower xylooligosaccharides (Bailey et al. 1992; Sunna and Antranikian 1997). Endoxylanase (1,4-β-D-xylan xylanohydrolase; EC 3.2.1.8), β-xylosidase (1,4-β-D-xylan xylohydrolase; EC 3.2.1.37), α-glucuronidase (EC 3.2.1.139), α-L arabinofuranosidase (EC 3.2.1.99), and acetyl xylan esterase (EC 3.1.1.6) represent the prominent enzymes in xylanolytic enzyme system. Among these, endoxylanase and β-xylosidase are considered as the crucial xylanolytic enzymes that act on xylan backbone for depolymerizing it into lower xylooligosaccharides (XOs). Endoxylanases cleave the glycosidic bonds and liberate various XOs, while β-xylosidase releases only xylose residues as an end product by acting on nonreducing ends of xylobiose/xylooligosaccharides (Motta et al. 2013). Acetyl xylan esterase, glucuronidase, ferulic esterase, and arabinosidase are other associated xylanolytic enzymes that remove the respective side chains from the xylan backbone (Dhiman et al. 2008). Microorganisms have always been a rich source of xylanolytic enzymes that include prokaryotic (bacteria and archaea) as well as eukaryotic microorganisms (yeast, fungi, and algae). Fungi and bacteria, however, are considered as the main source of industrially relevant xylanolytic enzymes due to their properties that meet the needs of various industrial downstream processes (Mandal 2015). Bacterial xylanases are preferred over other xylanases (Kumar et al. 2017), because they offer a broader range of xylanases with high substrate specificity, broad pH/and temperature tolerance ranges, and high stability at extreme pH and temperatures. This review thus focuses on xylanases of prokaryotic microbes either from culturable ones or from metagenomes.

14.2 Why Prokaryotic Xylanases?

Xylanases are produced by both bacteria and archaea. Bacteria have been well exploited for the production of xylanolytic enzymes in comparison with archaea. Xylanases produced by bacteria and actinobacteria are active in a broad range of pH (5.0–10.0) and temperature (30–80 °C) [Beg et al. 2001; Mandal 2015; Motta et al. 2013]. Fungal xylanases exhibit higher activity as compared to those of bacteria or yeast. Of these, filamentous fungi such as *Thermomyces*, *Aspergillus*, *Fusarium*, *Myceliophthora*, *Penicillium*, and *Trichoderma* are known to produce xylanases extracellularly (Deswal et al. 2014; Guan et al. 2016; Ramanjaneyulu and Reddy 2016). However, these xylanases do not suit well because of their limited tolerance to the harsh treatments in various industrial processes such as paper and pulp industry (Mandal 2015; Robledo et al. 2016). The paper and pulp industry demands xylanases having stability at higher alkaline pH. Majority of fungal xylanases display optimum activity in acidic range of pH (Polizeli et al. 2005; Ramanjaneyulu and Reddy 2016; Robledo et al. 2016). Recently, a xylanase from a fungus *Cladosporium oxysporum* GQ-3 has been reported to exhibit optimum activity at pH in the alkaline range (pH 8.0); this protease-resistant xylanase was optimally active at 50 °C. Therefore, this does not suit well for paper and pulp industry (Guan et al. 2016). Cellulase-free xylanase is another significant parameter for its use in paper and pulp industry to protect the cellulosic fibers from cellulases. Several fungal xylanase preparations display cellulase activity that limits their use in the paper industry (Subramaniyan and Prema 2002; Polizeli et al. 2005). Short generation time, harvesting steps, and enzyme yields are the crucial factors in the overall cost of an enzyme.

Archaea represent another domain of prokaryotes, which are known to be a source of extremozymes because of their occurrence in extreme environments (Eichler 2001). Hemicellulases have been shown to be produced by archaea. Bragger et al. (1989) were the pioneers in reporting putative hemicellulase activity in two uncharacterized strains of *Thermofilum*. Three archaeal stains were observed to produce xylanase (Uhl and Daniel 1999). Of the three, *Thermococcus zilligii* strain AN1 was extensively characterized for its endoxylanase activity. Endoxylanase was optimumally active at pH 6.0 with a half-life of 8 min at 100 °C along with broad substrate specificity (Uhl and Daniel 1999). Majority of archaea are not able to hydrolyze native xylan, thus require pretreated xylan as a carbon and energy source (Sunna and Antranikian 1997). Various members of Crenarchaeotes have been isolated such as *Thermosphaera aggregans* (Huber and Stetter 1998), *Sulfolobus solfataricus* (Cannio et al. 2004), and *Acidilobus saccharovorans* (Prokofevaetal, 2009) by incorporating autoclaved xylan into the culture medium. With an improvement in traditional microbiology, a few archaeal strains have been isolated in recent years which are capable of utilizing untreated native xylan as a carbon source and reveal their ability to secrete xylanases into the culture medium (Kublanov et al. 2009; Gavrilov et al. 2016; Menasria et al. 2018). Gavrilov et al. (2016) isolated a euryarchaeon, *Thermococcus* sp. strain 2319x1 that grew solely on untreated xylan. In a recent study, seven phylotypes of Haloarchaea were found to show various

hydrolase activities. Among all, *Haloferax*, *Halococcus*, *Halogeometricum*, *Haloterrigena*, *Natrialba*, and *Haloarcula* exhibited extracellular xylanase activity (Menasria et al. 2018). Further investigations are called for finding xylanases of Archaea. Of the characterized archaeal xylanases, the majority of them exhibit optimum pH in acidic range; there is no report till date on alkaline xylanases. In addition, a few of them showed cellulase activity that make them out from paper and pulp industry access. There is a long way to bridge the legitimate gap between the existing archaeal xylanases and the xylanases of industrial demand.

14.3 Enzymes Involved in Xylan Degradation

Xylans in gymnosperms and softwood are homopolymers of xylosyl residues that require only endoxylanase and β-xylosidase for their complete depolymerization. Heterogenous nature of xylans present in the environment from plant sources requires the synergistic action of a group of xylanolytic enzymes for the complete degradation of a heteroxylan (Kumar et al. 2013a, b). Therefore, a xylanolytic system includes β-1,4-endoxylanase, β-xylosidase along with the side chain acting enzymes such as α-L-arabinofuranosidase, α-glucuronisidase, acetyl-xylan esterase and phenolic acid esterases. In addition, β-galactosidase, β-glucuronidase and α-1,3-xylanases also contribute to the xylanolytic systems. The entire group of xylanolytic enzymes makes a consortium of xylanolytic system which is widely present in bacteria, fungi, archaea, and uncultured microorganisms (Woodward et al. 1984; Wong et al. 1988; Coughlan and Hazlewood 1993; Dekker and Richards 1976). The mode of action of various enzymes in multi-enzyme xylanolytic systems is described below.

14.3.1 Endoxylanase [D-Xylan Xylohydrolase (EC 3.2.1.8)]

Endoxylanase is the most significant enzyme in xylan hydrolysis. This enzyme directly attacks the glycosidic bonds between the xylosyl residues in xylan. It leads to reduction in the degree of polymerization (DP) which chiefly relies on the nature of xylan to be attacked. Heteroxylans take longer time to achieve the same level of reduction in DP than homo-xylans. A decrease in DP of xylan depends on the presence of attached side groups with the backbone, their lengths, and frequency of branching (Reilly 2014). The catalytic action of endoxylanase results in the formation of heterogenous mixture of high and low molecular weight xylooligosaccharides. Prolonged exposure to endoxylanase leads to depolymerization of high molecular weight XOs into lower XOs. XOs like xylotetraose, xylotriose, and xylobiose are formed by the action of endo-acting xylanases (Wong et al. 1988; Dekker and Richards 1976). Arabinose, glucuronic acid, and furfurals are also released from endoxylanase action. Endoxylanases are further classified as arabinose-liberating and non-arabinose-liberating types (Reilly 2014; Dekker and Richards 1976).

The dedicated families of endoxylanases are glycosyl hydrolase families 10 and 11 based on amino acid sequence similarity. GH-5, GH-8, and GH-141 have also been introduced to include xylanases which act on the glycosidic bonds other than β-1,4 linkages.

14.3.2 β-D-Xylosidase (β-D-Xyloside Xylohydrolase; EC 3.2.1.37)

β-D-Xylosidase, an exoglycosidase that acts on the non-reducing end of the lower XOs and xylobiose (Wong et al. 1988). It may be considered as a secondary enzyme of the xylanolytic system due to its catalytic action on the end products of endoxylanases. In laboratories, true β-D-xylosidase is identified by their reaction with the artificial substrate p-nitrophenyl β-D-xyloside that gives a yellow color complex. Thin-layer chromatography (TLC) is another popular method for identifying the true action of β-D-xylosidases which appear as an intense spot against the standard of commercial xylose. The β-D-xylosidases are high molecular weight monomeric or dimeric proteins having molecular mass in the range of 60–360 kDa and abundantly present in bacteria, yeast, and fungi. Majority of β-D-xylosidases are cell bound due to their action to form a monomeric form of sugar that can be directly transported into the cytoplasm using intermembranous β-D-xylosidases (Joseleau et al. 1992). Extracellular β-xylosidase has also been reported from bacteria and fungi, where xylose sugar may be transported into the cytoplasm by undefined mode of action (Dekker and Richards 1976; Zimmermann et al. 1988; Dobberstein and Emeis 1991). β-Xylosidases have been categorized into GH-1, GH-3, and GH-120 having the three-dimensional structure of $(\beta/\alpha)_8$ type. β-D-Xylosidases are known for end product inhibition due to the liberation of their hydrolysis product xylose. The enzyme has several applications besides the recovery of xylose for pharmaceutical industries. It can be used in de-inking of the waste paper along with the endoxylanases (Marques et al. 2003). D-Xylose derived from β-D-xylosidases action can be biotransformed into sugar alcohol known as xylitol, which exhibits natural sweetness and is used in improving dental health (Tamburini et al. 2015).

14.4 Emerging Role of Lytic Polysaccharide Monooxygenase in Xylan Degradation

Lytic polysaccharide monooxygenases (LPMOs) are copper-dependent enzymes that break the glycosidic bonds of plant polysaccharides. The LPMOs act in the early stage of plant cell wall degradation, where it boosts plant polysaccharide degradation by GHs (Harris et al. 2010; Xu et al. 2012; Hu et al. 2014). Several microorganisms from bacteria to fungi have been reported to produce LPMOs. In CAZy database, there are six auxiliary activity (AA) families which include LPMOs based on their amino acid sequence. Previously LPMOs were classified as glycosyl hydrolases (GHs) because of their weak endocellulase activities (Karlsson et al. 2001; Karkehabadi et al. 2008). LPMOs containing families belong to AA9 (formerly

GH61), AA10 (formerly CBM33), AA11, AA13, AA14, and AA15 (Lombard et al. 2014; Couturier et al. 2018; Sabbadin et al. 2018). The AA9 family exclusively contains enzymes of fungal origin, while AA10 family is dominated by bacterial LPMOs and then viral origin LPMOs. The AA11, AA13, and AA14 mainly deal with fungal LPMO sequences, while AA15 is a new addition to AA which groups LPMOs of eukaryotic origin, and interestingly it also holds LPMOs of insect origin. Putative AA10 proteins are mostly present in *Proteobacteria*, *Actinobacteria*, and *Firmicutes* (Paspaliari et al. 2015). Screening of bacterial genome for LPMOs suggested that bacteria typically contain AA10 protein encoding genes that occur in the range of 1–7, which is comparatively lesser than the fungal genomes, where up to 50 LPMOs from AA9 family could be found (Horn et al. 2012; Levasseur et al. 2013). This high number of LPMOs in fungal genomes has not been studied in detail till date. Many investigations have shown upregulation of genes from AA9 family in many species upon growth on cellulose (Foreman et al. 2003; Vanden et al., 2010). It clearly indicates the significance of this class allowing microorganisms to utilize the full calorific content of polysaccharides. LPMOs depolymerize a variety of polysaccharides. LPMO of AA10 was first discovered from *Serratia marcescens* as chitin-degrading LPMO, while AA9 from *Thermoascus aurantiacus* was a cellulose-degrading LPMO (Vaaje-Kolstad et al. 2010; Quinlan et al. 2011). Further, AA9 and AA10 also depolymerize starch, cellodextrins, xyloglucan and glucomannan, and cellulose-bound xylan (Vu et al. 2014; Borisova et al. 2015; Agger et al. 2014; Bennati-Granier et al. 2015; Frommhagen et al. 2015). Till now, there is no report of LPMOs acting specifically on isolated xylan. However, the presence of LPMOs in diverse taxa suggests a broad substrate range. Factors determining the substrate specificity of LPMOs are poorly understood because of indirect methods for determining the enzyme chemistry and complex structural and electronic factors. LPMOs depolymerize lignocellulose through an oxidative mechanism which involves hydroxylation of cellulose at the C_1 or C_4 carbon. LPMOs remove the hydrogen atoms and cleave the β-1,4-glycosidic bonds between C_1 and C_4 of the cellulose. This reaction produces aldonic acids and/or ketoaldolases on oxidation of glucose units of cellulose at C_1 and C_4 position, respectively. Breakdown of cellulose by LPMOs requires an extracellular source of electron and molecular oxygen. Small molecular reductants present in lignocellulosic biomass such as cellobiose dehydrogenase (CDH), plant and microbial phenols, pyrogallol, ascorbate, gallic acid, or sulfur-containing species may act as extracellular source of electron donors. Electron donors activate the action of LPMOs. Cellobiose dehydrogenases (CDH) and LPMOs cooperate to form an oxidation system, while other electron-donating groups observed in literature are gallic acid, sulfur-containing species, ascorbate, and photosynthetic pigments. This oxidative cleavage of cellulose by LPMOs is different from hydrolytic cleavage employed by GHs (Hemsworth et al. 2013; Walton and Davies 2016). Common structural feature of all LPMOs is an immunoglobulin-like structural core with a flat substrate-binding surface. X-ray crystallography revealed that LPMOs have single copper ion where catalysis happens; copper ion is coordinated by a histidine brace. A rare motif "histidine brace" is conserved across all LPMOs, whereas the secondary coordination sphere of the

catalytic copper and the rest of the substrate-binding region are quite diverse (Quinlan et al. 2011). In AA10, motif "1" or loop "2" of the β-strand is the most diverse region, and it consists of many short helices and loops (Wu et al. 2013; Book et al. 2014). It is believed that "loop 2" of the β-strand influences substrate identification and specificity (Wu et al. 2013; Book et al. 2014; Borisova et al. 2015).

LPMOs have been discovered recently and need extensive research in understanding their diversity and catalytic potentials for industrial applications. The addition of LPMOs to commercially available cellulase cocktails reduces the required enzyme dose by twofolds (Harris et al. 2010). When one LPMO from AA10 family of CAZy database engineered to form a designer cellulosome along with two other cellulases, this designer cellulosome exhibited a 1.seven-fold increase in glucose yield as compared to the unassembled enzymes of the same combination (Arfi et al. 2014; Hemsworth et al., 2015). These findings are interesting and encouraging researchers to explore more about LPMOs (Beeson et al. 2015; Morgenstern et al. 2014).

14.4.1 α-Glucuronidase (EC 3.2.1.139)

Xylan α-1,2-glucuronidase is a side chain-acting enzyme of the xylanolytic system, which cleaves α-linked glycosyl bonds in between the glucuronic acid and/or methyl-glucuronic acid and xylosyl residues of glucurono-xylan. The CAZy database classifies α-glucuronidases into glycosyl hydrolase families 67 and 115 having the 3D structure of $(\beta/\alpha)_8$ type (Rogowski et al. 2014). The GH-67 glucuronidases remove uronic acids present at the nonreducing end of glucuronic xylooligosaccharides, while GH-115 has a broader mode of action and covers both GlcA/MeGlcA xylan and glucurono-xylooligosaccharides for liberating uronic acid (Ryabova et al. 2009). α-1,2-Glucuronidase has been reported from several bacteria such as *Bacillus stearothermophilus* T-6 (Zaide et al. 2001), *Cellvibrio japonicus* (Nagy et al. 2003), *Geobacillus stearothermophilus* (Golan et al. 2004), and *Streptomyces pristinaespiralis* (Fujimoto et al. 2011). The exclusively decorated 2-O-glucuronic acid and/or 2-O-methyl-glucuronic acid on xylan backbone inhibits the depolymerization by protecting it from the direct attack of most of the endoxylanases and β-xylosidases (Gilbert 2010; Gilbert et al. 2008). Therefore, side chain-acting enzymes like α-1,2-glucuronidases are needed to overcome this bottleneck. Recently, one α-1,2-glucuronidase of family GH-115 has been reported from a marine bacterium *Saccharophagus degradans* 2–40 that exhibited NaCl activation (Wang et al. 2016). At present, CAZy database (updated on May 17, 2018) of GH-115 has a total of 116 glucuronosidases [α-1,2-glucuronidase and α-(4-O-methyl)-glucuronidase], of which 357 are of the bacterial type and 4 show their origin from archaea. Among all, 11 glucuronosidases have been retrieved from an uncultured microorganism. Similarly, GH-67 contains 578 glucuronidases, where the bacterial count is 535 and 4 are of archaeal origin. More investigations are called for characterizing glucuronidases.

14.4.2 Arabinofuranosidase

Arabinofuranosidases or AFases catalyze α-linked L-arabinofuranose residues at the nonreducing end of the arabinan attached with xylan backbone (Raweesri et al. 2008; Kumar et al. 2013a, b). AFases occur in nature in two different forms: first is exo-AFase, α-L-arabinofuranosidase (EC 3.2.1.55) that acts on branched arabinans and p-nitrophenyl-α-L-arabinofuranosides, while the second one is endo-acting 1,5-α-L-arabinase (EC 3.2.1.99) that hydrolyzes branched arabinans. These anchored xylans demand synergistic scion of AFase along with endoxylanase. AFase action makes the heteroxylan accessible for endoxylanases. There are six GH families, viz., GH-43, GH-62, GH-127, GH-137, GH-142, and GH-146, which include members of arabinofuranosidase. GH-62 exclusively contains α-L-arabinofuranosidases, while GH-127, GH-137, GH-142, and GH-146 exhibit β-L-arabinofuranosidase as one of the enzymes. Several bacteria have been reported to produce α- and β-L-arabinofuranosidases (Kaji and Saheki 1975; Kaji 1984; Kurakake et al. 2014). An acidic arabinofuranosidase from *Streptomyces* sp. I10–1 has been reported to efficiently liberate arabinose sugars from corn hull arabinoxylan at 40 °C (Kurakake et al. 2014). Recently a gene encoding L-arabinofuranosidase from *Streptomyces* sp. SWU10 has been cloned and overexpressed in order to achieve the arabinoxylan degradation (Phuengmaung et al. 2018). On characterization, this GH-62 enzyme showed optimum pH and temperature at 5 and 50 °C, respectively (Phuengmaung et al. 2018). Synergistic action of endo- and exo-acting arabinofuranosidases enhances the production of XOs and arabinose sugars. L-Arabinose has application in pharmaceuticals and is used in food industry as a flavoring agent, food ingredient, and dietary supplement.

14.4.3 Acetyl Xylan Esterases (EC 3.1.1.72)

Acetylation and feruloylation of the xylan protect the plant from the direct access of majority of lignocellulases (Rennie and Scheller 2014). Acetyl xylan esterases, therefore, are required to assist in xylan hydrolysis. Acetyl xylan esterases (AXEs) have been reported for the first time in microorganisms during 1985 (Joseleau et al. 1992). This side chain-acting enzyme catalyzes the hydrolysis of an acetyl group from acetylated xylose, acetylated glucose, polymeric xylan, triacetyl glycerol, α-naphthyl acetate, and p-nitrophenyl acetate (Biely et al. 2016; Nakamura et al. 2017). Acetyl xylan esterase has not, however, been reported for its activity toward acetylated pectin or mannan. Acetyl xylan esterases are grouped into carbohydrate esterase family from CE-1 to CE-7 and CE-12 of carbohydrate esterases (CEs) of CAZy database. Several AXEs have been reported from bacterial sources (Adesioye et al. 2016; Nakamura et al. 2017). A novel AXE has been reported in *G. stearothermophilus* T-6 that belongs to glycine-serine-aspartate-lysine (G-S-D-L) hydrolase family and does not show any sequence homology with any of the available sequences in the database (Alalouf et al. 2011). Recently, *Flavobacterium johnsoniae* has been shown to have a cluster of acetyl xylan esterases, which were

categorized into CE-1 to CE-6 families of AXEs (Razeq et al. 2018). AXE has been successfully employed for enhancing the solubilization of xylan from wheat straw and giant reed (Zhang et al. 2011). Several AXEs have been reported to date, where most of them are of the bacterial type, which need characterization.

14.5 CAZy View of Xylanolytic Enzymes

The carbohydrate-active enzyme (CAZy) database was established in 1998 to categorize the collective information of carbohydrate-active enzymes (www.cazy.org). It classifies all the polysaccharide-relevant enzymes into five broad classes, viz., glycosyl hydrolases (GHs), glycosyl transferases (GTs), polysaccharide lyases (PLs), carbohydrate esterases (CEs), and auxiliary activities (AAs). Of the five, xylanolytic enzymes belong to GH- family. This categorization relies on amino acid sequence similarities. At present, xylanolytic enzymes fall into 20 different families of GHs. It includes GH-1, GH-3, GH-5, GH-8, GH-10, GH-11, GH-16, GH-26, GH-30, GH-43, GH-62, GH-67, GH-74, GH-115, GH-120, GH-127, GH-137, GH-141, GH-142, and GH-146 (Table 14.1). Among all, GH-10 and GH-11 families exhibit majority of the endo-acting xylanases. At present, GH-10 family contains 3213 xylanases including tomatinase from various sources, where 2438 belong to bacterial and 19 account for archaeal types. The signature sequence of GH-10 (cellulase family F) contains glutamate as a catalytic nucleophile/base and the proton donor. Another prominent family of endoxylanase belongs to GH-11, i.e., cellulase family G. The CAZy database of GH-11 comprises 1473 xylanase proteins (endo-β-1,4-xylanase and endo-β-1,3-xylanase). Of which, maximum xylanases (851) are of bacterial origin, while 6 of archaeal and approximately 107 are unclassified xylanases. The total number of extensively characterized xylanases from GH-10 and GH-11 family accounts for approximately 624, where 352 are of GH-10 and the remaining 272 belong to GH-11-type xylanases.

β-Xylosidases belong to one of the most abundant xylanolytic enzymes that produce xylose as an end product from XOs and xylobiose. CAZy classifies the β-xylosidases into five different families, viz., GH-1, GH-3, GH-30, GH-43, and GH-120, where GH-43 includes the highest number of β-xylosidase protein sequences having aspartate and glutamate residues in their catalytic domains. Glycosyl hydrolase family 43 further classifies enzymes (β-xylosidase, α-L-arabinofuranosidases, α-L-arabinanases, β-D-xylosidases, xylanase, and β-D-galactosidases) into 37 subfamilies where all exhibit the common fivefold β-propeller structure. The α-L-arabinofuranosidases also share a significant chunk of the xylanolytic system that assists in catalyzing the cleavage of glycosidic bonds of arabinoxylan and arabinogalactans. The CAZy database has six families having α-L-arabinofuranosidases, namely, GH-43, GH-62, GH-127, GH-137, GH-142, and GH-146. Other side chain-acting enzymes of xylan such as α-glucuronidase, β-galactosidase, acetyl xylan esterase, and phenolic acid esterases have also been categorized into various families of GHs. The CAZy database is expanding day by day; the present defined report is based on the latest update of the database on May 17, 2018.

Table 14.1 Prokaryotic and other unclassified xylanolytic enzymes present in various families of CAZy database

Family	Representative xylanolytic enzymes*	3D structure	Catalytic nucleophile base	Catalytic proton donor	Archaea	Bacteria	Unclassified/ metagenomic
GH-1	β-Xylosidase	$(\beta/\alpha)_8$	Glutamate	Glutamate	143	18,969	49
GH-3	β-Xylosidase	–	Aspartate	Glutamate	102	17,820	101
GH-5	Endo-β-1,4-xylanase	$(\beta/\alpha)_8$	Glutamate	Glutamate	92	9308	120
GH-8	Endo-β-1,4-xylanase	$(\alpha/\alpha)_6$	Aspartate**	Glutamate	–	3497	18
GH-10	Endo-β-1,4-xylanase, endo-β-1,3-xylanase	$(\beta/\alpha)_8$	Glutamate	Glutamate	19	2438	356
GH-11	Endo-β-1,4-xylanase, endo-β-1,3-xylanase	β-Jelly roll	Glutamate	Glutamate	06	851	107
GH-16	Xyloglucan: Xyloglucosyltransferase	β-Jelly roll	Glutamate	Glutamate	22	3723	13
GH-26	β-1,3-Xylanase	$(\beta/\alpha)_8$	Glutamate	Glutamate	08	1519	17
GH-30	Endo-β-1,4-xylanase; β-xylosidase	$(\beta/\alpha)_8$	Glutamate	Glutamate	–	1762	02
GH-43	β-Xylosidase; xylanases; α-1,2-L-arabinofuranosidase	$(\beta/\alpha)_8$	Glutamate	Glutamate	19	9482	28
GH-62	α-L-Arabinofuranosidase	ND	ND	ND	–	221	03
GH-67	Xylan α-1,2-glucuronidase	$(\beta/\alpha)_8$	ND	Glutamate	04	535	11
GH-74	Xyloglucanase	7-fold β-propeller	Asp	Asp	–	383	03
GH-115	Xylan α-1,2-glucuronidase	ND	ND	ND	04	537	11

GH-120	β-Xylosidase	ND	Aspartate**	Glutamate **	–	144	–
GH-127	β-L-Arabinofuranosidase	$(\alpha/\alpha)_6$	ND	ND	7	1554	–
GH-137	β-L-Arabinofuranosidase	5-fold β-propeller	Glu	Glu	38	–	–
GH-141	Xylanase	(β)-helix	Asp	Asp	–	188	–
GH-142	β-L-Arabinofuranosidase	$(\alpha/\alpha)_6$	Asp	ND	–	70	–
GH-146	β-L-Arabinofuranosidase	$(\alpha/\alpha)_6$	ND	ND	–	559	–

*Groups may contain carbohydrate-hydrolyzing enzymes other than xylanolytic enzymes; **Inferred catalytic residues

14.6 Classification of Xylanases

The CAZy database follows the sequence similarity for classifying various hydrolases, as common amino acids turn out with similar structures and grooves (www.cazy.org). Stretches of similar or/and identical amino acids share the same conserved domains such as carbohydrate-binding domains and catalytic clefts. It further assists to trace out the evolutionary changes and assigning phylogenic relationships with the available or new enzyme entries in a database. Glycosyl hydrolases of related xylanolytic enzymes have been categorized by CAZy database into 20 different families which have further sublevel classifications according to their specific properties. Signature sequences of glycosyl hydrolases have revealed that aspartate and glutamate share the catalytic nucleophile/base and proton donor in the majority of the xylanolytic enzymes to attack glycosidic bonds of various xylan types (Fig. 14.1). However, structural variation occurs in xylanolytic enzymes (Table 14.1); GH-10 xylanases are of $(\beta/\alpha)_8$ type, while GH-11 xylanase exhibits β-jelly roll-shaped structure. GH-8 and GH-10 xylanases share the close proximity in architecture $(\beta/\alpha)_6$ as well as in the substrate specificity (Collins et al. 2002). In addition, $(\alpha/\alpha)_6$, five-fold β-propeller and seven-fold β-propeller only β-helices exist in various side chain-acting enzymes of the xylanolytic system (Table 14.1). Besides this broad classification, one more classification discusses the categorization of xylanases which is based on their physiological properties. These properties chiefly include molecular weight and isoelectric points, i.e., pI values. Based on this,

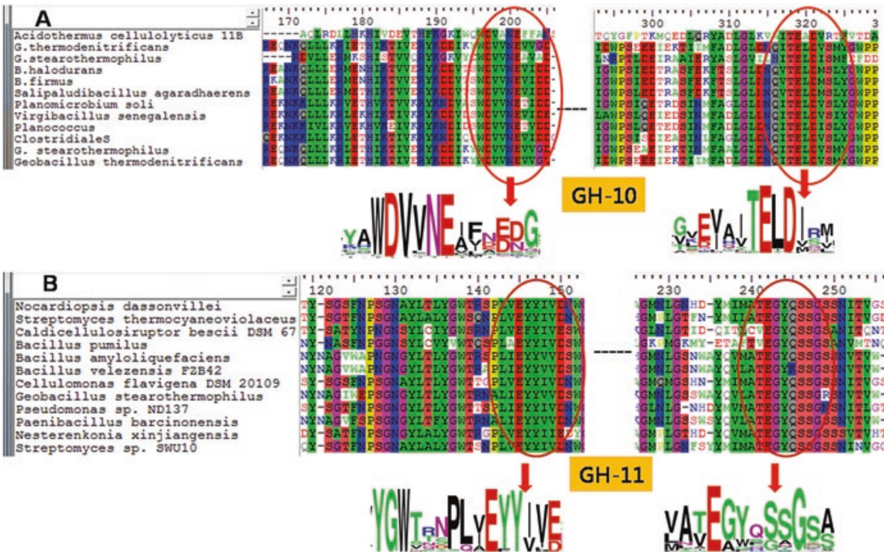

Fig. 14.1 The ClustalW multiple alignment shows the consensus sequences having catalytically important residue (Glutamate; E) in bacterial xylanases of GH-10 (**a**) and GH-11 (**b**) family. The logos indicate conserved amino acid residues, where the height of each amino acid reflects conserved nature

xylanases of high molecular weight (> 30 kDa) and acidic pI are grouped into glycosyl hydrolase family 10 (cellulase family F), while low molecular weight xylanases having basic isoelectric point belong to glycosyl hydrolases family 11 (cellulase family G) (Collins et al. 2005; Verma and Satyanarayana 2016). However, several exceptional xylanases exist in nature from microorganisms that contradict this rule and go well with the CAZy-based classification. Xylanase of *Cellulomonas flavigena* CDBB-531 has a molecular weight of 35 kDa, although it belongs to GH-11 family (Amaya-Delgado et al. 2010). Similarly, *Clostridium stercorarium* NCIMB 11754 also produces high molecular weight exceptional xylanases of GH-11 family (Adelsberger et al. 2004; Berenger et al. 1985). Metagenomic xylanase, Mxyl from a metagenome, has also been characterized as a member of GH-11 family despite its high molecular weight of ~ 40 kDa (Verma et al. 2013a). Furthermore, many xylanases do not fit well into this mode of classification due to their unusual properties (Sunna and Antranikian 1997). GH-10 hydrolyzes the internal β-1,4-glycosidic bonds of the heteroxylan backbone along with aryl-D-xylosidase activity. GH-10 and GH-11 xylanases are the only groups having xylanases that act on the backbones of the hetero−/homo-xylans.

14.7 Classification Based on Temperature for Activity

Based on the optimum temperature for the activity of enzymes, xylanases have also been classified into psychrophilic (< 20 °C), mesophilic (25–45 °C), moderately thermophilic (50–70 °C), and hyperthermophilic (>80 °C) enzymes. A wide range of microorganisms has been reported to produce the xylanases of various physiological properties (Chakdar et al., 2016; Walia et al. 2017; Guan et al. 2016; Heinze et al. 2017). Arrhenius law states that thermophiles are the better source for discovering thermostable enzymes as compared to the enzymes of mesophilic origin, because at higher temperatures, enzymes get adapted to act at elevated temperatures (Collins et al. 2005). In our laboratory, several thermophilic bacteria have been isolated from extreme environments while looking for thermoalkalistable xylanases. These include *Bacillus licheniformis* A99 (Archana and Satyanarayana 1997), *Geobacillus thermoleovorans* AP07 (Sharma et al. 2007), *G. thermoleovorans* (Verma and Satyanarayana 2012b), *Bacillus halodurans* TSEV1 (Kumar and Satyanarayana 2011), and *Geobacillus thermodenitrificans* TSAA1 (Verma et al. 2013b). Xylanases produced by these thermophilic bacteria follow the Arrhenius law as all showed optimum activities in the range of 60–80 °C at alkaline pH. A total of 31 xylanases have been reported from 161 thermophilic *Bacillus* species from various hot springs of Tunisia (Thebti et al. 2016). Based on 16S rDNA sequence analysis, various *Bacilli* have been identified as *Geobacillus kaustophilus*, *Aeribacillus pallidus*, *Geobacillus galactosidasus*, and *Geobacillus toebii*. A hydrothermal vent of Algeria revealed a xylanase from a thermophilic anaerobic bacterium *Caldicoprobacter algeriensis* having optima of pH 11.0 and 70 °C (Amel et al. 2016). One metagenomic GH-11 xylanase (Mxyl) from a high-temperature compost plant, Fukuoka, Japan, has also reflected similar properties having T_{opt} of 80 °C

(Verma et al. 2013a). Similarly, a xylanase (XynA) obtained from hot pool metagenomic library displayed optimum activity at 100 °C (Sunna and Bergquist 2003). Volcano crater has also been a source of a thermostable xylanase (T_{opt}, 95 °C) from its metagenome (Mientus et al. 2013). Traditional cultivation approaches are always preferred over the metagenomic approaches due to the hassle-free cultivation on well-defined media and chromogenic screening for the respective biomolecules. By this approach, less than 1% of microorganisms come in a grab, although their count is much higher than those from metagenomics. Several species of *Bacillus*, *Clostridium*, *Actinomadura*, *Thermoactinomyces*, and *Dictyoglomus* have been reported to produce thermostable xylanases (Kumar et al. 2013a, b). *Rhodothermus marinus* ITI376 (Dahlberg et al. 1993), *Bacillus amyloliquefaciens* (Breccia et al. 1998), *G. thermoleovorans* AP07 (Sharma et al. 2007), *B. halodurans* TSEV1 (Kumar and Satyanarayana 2011), *G. thermodenitrificans* (Verma et al. 2013b), and *Stenotrophomonas maltophilia* (Raj et al. 2013) are a few bacteria which produce thermostable xylanases with optima at 80 °C. Majority of xylanases from *Bacillus*, *Geobacillus*, *Paenibacillus*, and other *Firmicutes* exhibits optima in the temperature range of 50 to 80 °C. The count of hyperthermostable xylanases of bacterial origin is handful which exhibit activity above 80 °C. Members of the genus *Thermotoga* are well known for the production of extremozymes due to their survival at or above 90 °C (Schroder et al. 1994). Extremely thermostable xylanases have also been reported from different *Thermotoga* spp. (Winterhalter and Liebl 1995; Yu et al. 2016). The pioneering work was done by Winterhalter and Liebl (1995), who discovered two highly thermostable and inducible endoxylanases, xynA and xynB, from *Thermotoga maritima* MSB8 having their optima at 92 and 105 °C, respectively (Winterhalter and Liebl 1995). Both these xylanases were cell wall-associated with signal peptide in the enzymes (Liebl et al. 2008). On expression of *xynB* gene in *E. coli*, the recombinant xynB showed a high affinity toward the oat-spelt xylan having Km value of 0.079 mg/ml with a maximum production of xylobiose as an end product (Jiang et al. 2004). Another thermostable xylanase from *Thermotoga neapolitana* showed significantly high thermostability having $T_{1/2}$ of 130 min at 82 °C, whereas its optimum activity was at 85 °C. (Bok et al. 1994). A xylanase (xyn10A)-encoding gene (3474 bp) from the hyperthermophilic bacterium *Thermotoga thermarum* was successfully cloned and expressed in a heterologous host *E. coli*. On characterization, it displayed optimum activity at 95 °C which further enhanced in the presence of 5 mM Ca^{2+} (Shi et al. 2013). An artificially synthesized gene of an endoxylanase (TmXYN10B) from *Thermotoga maritima* was successfully cloned and expressed in the yeast *Pichia pastoris*. The recombinant xylanase was optimally active at 100 °C, retaining 92% residual activity at 105 °C (Yu et al. 2016). *Thermotoga hypogeum* was found to have endoxylanase with optimum at 90 °C (Frock et al. 2010). It is remarkable that various species of *Thermotoga* share the common feature in their endoxylanases as all of them belong to GH-10 family and majority of them exhibit their optimum pH for activity in the acidic range (Winterhalter and Liebl 1995; Frock et al. 2010; Yu et al. 2016). None of the xylanases of GH-11 has been reported till date from *Thermotoga* spp., and none has shown optimal activity at alkaline pH. A similar endoxylanase had been reported

from *Acidothermus cellulolyticus* 11B having optima at 90 °C and pH 6.0 with a half-life of 90 min at 90 °C (Barabote et al. 2010). In presence of 0.5 M sorbitol, the xylanase displayed 6.five-fold higher thermostability. Polyols like mannitol, glycerol, and sorbitol act as stabilizers by enhancing the viscosity of the samples and protect the entrapped enzymes from the direct effect of temperature (Khandeparkar and Bhosle 2006; Bankeeree et al. 2014). A thermostable xylanase (optimum pH 9.0 and temp. 50 °C) had been discovered from a bacterium *Bacillus amyloliquefaciens* strain SK-3 isolated from soil samples, which is suitable for pulp bleaching (Kumar et al. 2017). Interestingly, a bacterium *Clostridium thermocellum* was found to have endoxylanase (Xyn141E) of GH-141 family. It showed optimum activity in the range of 67–75 °C at acidic pH in the range of 6.0–6.5 (Heinze et al. 2017). In recent years, several other xylanases from a broad range of bacteria have been reported (Basit et al. 2018). Thermostability and alkalistability of xylanases are the chief concerns of various industries, for which extreme environments have always been targeted. The ideal xylanases are handful; therefore, many of the available xylanases were engineered to make them fit for a process. DNA shuffling, directed evolution, protein engineering, incorporation of N- or C-terminal regions of thermostable enzymes, truncation of proteins, and rationale site-directed mutagenesis are the few approaches which have been employed successfully for generating industrially fit xylanases (Verma and Satyanarayana 2012a; Cheng et al. 2015; Booneyapakron et al. 2017; Basit et al. 2018; Kumar et al. 2017).

14.8 Classification on the Basis of pH Optima

Stability of xylanases at a particular pH decides its type and applicability in different industries. The pH significantly influences the three-dimensional structure of an enzyme and directly affects its enzymatic activity as well as substrate binding (affinity) [Collins et al. 2005]. Xylanases can be categorized into acidic, neutral, and alkaline types based on their optimum pH. Majority of fungal xylanases exhibit their optima in the acidic range, whereas xylanases of bacterial origin are scattered on a pH scale (Kumar et al. 2013a, b). Acidic bacterial xylanases dominate in the pH range of 5–6.5 having their applicability in baking industries and saccharification of agro-residues for producing lower xylooligosaccharides (Subramaniyan 2012; Bajaj and Singh 2010; Lin et al. 2017). Various species of *Bacillus* have been found to be sources of acidic xylanases, such as xylanase of *Bacillus circulans* D1 (Bocchini et al. 2002), *Bacillus pumilus* B20 (Geetha and Gunasekaran 2010), and *B. pumilus* SSP-34 (Subramaniyan 2012). In addition, other taxa of bacteria have also been reported to produce acidic xylanases. This includes xylanases from *Cellulomonas flavigena* (Santiago-Hernandez et al. 2007), *Streptomyces cyaneus* SN32 (Ninawe and Kuhad 2005), *Caldicellulosiruptor* spp. (Ying et al. 2013), *Thermosaccharolyticum* DSM 571 (Li et al., 2014), and *Pseudomonas* (Lin et al., 2017). Xylanases of *Caldicellulosiruptor* species (Ying et al. 2013), *Thermosaccharolyticum* DSM 571, and *Pseudomonas* (Lin et al. 2017) are also thermostable having their optima at 65 °C. Archaea are well known for their

thermostable acidic xylanases from various genera of *Sulfolobus* (Maurelli et al. 2008) and *Thermococcus* (Uhl and Daniel 1999). A range of neutral xylanases have been reported from the bacterial source; however, their applicability is less over acidic as well as alkaline xylanases (Kiddinamoorthy et al. 2008; Kui et al. 2010; Nawel et al. 2011). Alkalistable xylanases belong to the most significant category of xylanases due to their wide applications in various industries. Bacteria are the chief source of alkaline xylanases with a broad range of substrate specificity. Numerous bacteria from different genera are known to produce xylanases with optimum pH 8.0–11.0. *Firmicutes* is the most abundant taxa having xylanases of alkaline type, where *Bacillus* sp. Sam3 (Shah et al. 1999), *B. halodurans* S7 (Mamo et al. 2007), *Bacillus stearothermophilus* SDX (Dhiman et al. 2008), *Bacillus subtilis* (Annamalai et al. 2009), *B. halodurans* TSEV1 (Kumar and Satyanarayana 2011), and *G. thermoleovorans* (Verma and Satyanarayana 2012b) exhibit their optimum pH at 8.0 or above. Interestingly, all the xylanases mentioned here belong to the domain *Firmicutes* and are thermophilic in nature; therefore they can be employed in bleaching of pulp samples in paper industries. There is a scarcity of xylanases with optimum pH 9.0 and above along with fair stability. Xylanase of *Enterobacter* sp. MTCC 5112 (Khandeparkar and Bhosle 2006), *Thermobifida halotolerans* YIM 90462 T (Zhao et al. 2015), and *Stenotrophomonas maltophilia* (Raj et al. 2013) are a few names that produce alkaline xylanases showing their optimal activity at pH 9.0. A thermophilic bacterium *Actinomadura* sp. strain Cpt20 isolated from poultry compost showed extraordinary characteristics with optimum activity at 80 °C at highly alkaline pH of 10.0. Moreover, the xylanase showed a half-life of 2 h and 1 h at 90 °C and 100 °C, respectively (Taibi et al. 2012). Other members of *Actinomadura* isolated from compost soil have also shown very similar physical properties (Sriyapai et al. 2011). Various molecular techniques have also been adapted to alter the optimum pH of xylanases (Juturu and Wu 2012). In this context, alkaliphilicity of a xylanase from the strain *Bacillus* sp. SN5 was improved by 1 unit from 7.5 to 8.5 using random mutation and Glu135 saturation mutagenesis (Bai et al. 2015). Xylanases having stabilities at extreme pH (higher or lower) significantly enhance their demand in the industries. Moreover, thermostability at elevated temperatures makes them sturdy. Although there are very few reports on xylanases having twin stabilities, further efforts are called for finding novel enzymes or by modifying the available ones.

14.9 Categorization Based on the Mode of Action

Endoxylanases can also be categorized based on the type of catalytic mechanisms (Koshland Jr 1953; McCarter and Withers 1994). Depolymerization of xylan takes place by two well-defined mechanisms: the first follows the hydrolysis of glycosidic bonds with overall inversion of the anomeric configuration, while the second retains this configuration. Glutamate (E) is the only catalytically important residue that participates both nucleophile/base as well as proton donor in the xylanases of GH-5, GH-7, GH-10, GH-11 and GH-30 families and follows the retaining mechanism of

catalysis, while the xylanases of GH-8 and GH-43 families display inverting mode of catalytic action having aspartate (D) as nucleophile/base and glutamate as a proton donor. In both the mechanisms, the position of proton donor is constant, while that of nucleophile/base varies. Endoxylanases of retaining mechanism keep the nucleophile/base in close proximity to anomeric carbon of the sugar as compared to that of xylanases of the inverting mechanism (Koshland, 1953). This way inverting enzyme accommodates a water molecule in between the sugar and the participating nucleophile/base that almost doubles the distance from 5.5 to 10A° in inverting enzymes (McCarter and Withers 1994; Paes et al. 2012). Disturbances at the level of crucial and conserved amino acids alter the catalytically required distances by double displacement mechanisms and completely lose the enzyme activity (Shi et al. 2011). Site-directed mutagenesis has been successfully used to study the catalytic role of glutamate in xylanase action (Verma and Satyanarayana 2013c).

14.10 Xylanases from Bacteria

Firmicutes includes several xylanase-producing bacteria, where the count of bacilli is maximum followed by *Bacteroides*, *Caldicellulosiruptor*, *Dictyoglomus*, *Clostridium*, and several other members. Among bacilli, members of *Geobacillus* (Wang et al. 2013; Gerasimova and Kuisiene 2012; Biwi et al. 2015; Daas et al. 2017), *Paenibacillus* (Zheng et al. 2014), and *Bacillus* (Mamo et al. 2006) are well known for their xylanases from various hot environments. An acidic xylanase was characterized from a strain of *Geobacillus thermodenitrificans* (Gerasimova and Kuisiene 2012) that was optimally active at pH 6.0 and 70 °C, which is quite similar to the endoxylanase of *Geobacillus thermodenitrificans* T12 (Daas et al. 2017). Thermostability of this endoxylanase compromises at higher temperatures, and it is quite low in comparison with other xylanases of *Geobacillus*. A thermoalkalistable xylanase of *Geobacillus thermoleovorans* displayed $T_{1/2}$ of 60 min at 80 °C, while at 90 °C, it was 10 min (Sharma et al. 2007). Endoxylanase of *Geobacillus thermodenitrificans* JK1 was inhibited by Na^{+}, K^{+}, Ca^{2+}, and Co^{2+} (Gerasimova and Kuisiene 2012). Recombinant xylanase of *G. thermodenitrificans* TSAA1 followed a similar profile, where Hg^{2+}, Pb^{2+}, Cu^{2+}, and Pb^{2+} strongly inhibited xylanase activity (Verma et al. 2013b). Total inhibition of xylanase activity was recorded in presence of Hg^{2+}, indicating the existence of tryptophan residues in the catalytic groove that oxidized into the indole ring and disrupts the interactions (Zhang et al. 2007; Liu et al. 2010; Cheng et al. 2005; Gupta et al. 1992). Several *Paenibacillus* have been reported to display xylanase activity (Ko et al. 2010; Zheng et al. 2012a, 2014; Dheeran et al. 2012). *Paenibacillus* sp. NF1, another member of bacilli, exhibited high affinity toward oat-spelt xylan at 60 °C in slightly acidic condition (pH 6.0) (Zheng et al. 2014). In another report, *Paenibacillus campinasensis* G1-1 has been reported to produce a high titer of xylanase (xynG1-1) in a synthetic medium, where the maximum activity of xylanase was recorded at a pH 7.5 at 60 °C (Zheng et al. 2012a). *Bacillus megaterium* was found to overproduce xylanase that improves the brightness of cotton stalk pulp and saccharification of recycled waste paper (Zheng

et al. 2012b). A plethora of bacilli have been reported to produce broad-spectrum extracellular xylanases active in acidic to alkaline range (Kumar et al. 2013a, b; Kumar et al. 2016; Khandeparker et al. 2017). In an early report, an alkalophilic strain *Bacillus* NG-2 has been reported to secrete a thermostable xylanase having $T_{1/2}$ of 75 min at 70 °C (Gupta et al. 1992). Besides, it exhibited multiple xylanolytic enzymes which were confirmed as multiple bands on zymogram analysis (Gupta et al. 2000). A similar cellulase-free endoxylanase was reported from *B. licheniformis* A99 with maximum activity at 60 °C in the neutral range (Archana and Satyanarayana 1997). A highly alkaliphilic endoxylanase (pH 9.0) was characterized from *B. stearothermophilus* T-6 with T_{opt} at 65 °C (Khasin et al. 1993). Irfan et al. (2016) reported two species of *Bacillus* (*B. subtilis* and *B. megaterium*) to produce extracellular xylanases in submerged fermentation. Characterization of the respective xylanases from both sources revealed an optimum temperature of 50 °C under acidic conditions. Endoxylanase of *Bacillus cereus* also showed optimum activity at acidic pH of 6.0 at 40 °C (Roy and Rowshanul Habib 2009). Another mesophilic xylanase was reported from a new thermoalkalophilic species of *Bacillus* having an optimal temperature of 50 °C (Kable and Jhadave 2012). Xylanases of similar properties were reported from various species of *Aeromonas* (Ohkoshi et al. 1985) and *Cephalosporium* (Kang et al. 1996). Recently an alkalitolerant bacterium *Bacillus subtilis* ADI1 has been reported to produce extracellular xylanopectinolytic activity (Nawawi et al. 2017). Xylanases of different strains of *Bacillus halodurans* have been extensively characterized thermoalkalistable endoxylanases, where these xylanases display high activity at alkaline pH of 9.0–10.0 at 90–98 °C (Kumar and Satyanarayana 2011; Mamo et al. 2007). *Caldicellulosiruptor* belongs to anaerobic, gram-positive *Firmicutes* that harbors one of the most thermophilic, cellulolytic, and xylanolytic bacteria to date (Zhang et al. 2016). Morris et al. (1999) reported four xylanases from extremely thermophilic *Caldicellulosiruptor* sp. strain Rt69B.1, where three (xynA, xynB, and xynC) were of GH-10- and one (xynD) of GH-11-type xylanase. On sequencing, deduced molecular masses of xynB and xynC were categorized as high molecular weight modular xylanases (Morris et al. 1999). A close relative of *Caldicellulosiruptor saccharolyticus* DSM 8903 has been isolated from bio-compost that possessed two thermostable xylanases having $T_{1/2}$ of more than 4 h at 75 °C (Ying et al. 2013). An enzyme cocktail of six xylan-degrading enzymes from *Caldicellulosiruptor bescii* was proposed by Su et al. (2013). Here, all the six genes have been expressed in *E. coli* and characterized as acidic (5.5 to ~6.5) and thermostable (75 to ~90 °C). Very similar optimum properties (pH 7.2 and temperature 70 °C) were identified from another GH-10 xylanase of *C. bescii* (An et al. 2015). Recently, GH-10 xylanase (CbXyn10B) of *C. bescii* has been crystallized for its extensive characterization due to its unique properties (Zhang et al. 2016). *Clostridium* spp. produce thermostable xylanases (Grepinet et al. 1988; Zverlov et al. 2005). *Clostridium thermocellum* (*Ruminiclostridium thermocellum*) is an extensively exploited bacterium for studying lignocellulose-degrading enzymes (Hayashi et al. 1999; Zverlov et al. 2005; Heinze et al. 2017). *Clostridium* spp. harbor a cluster of extracellular hydrolytic enzymes known as cellulosomes for efficient degradation of the lignocellulosic biomass. Majority of

cellulosomal components are of β-glucanase type, and GH-10 and GH-11 xylanases are the other crucial constituents of the cellulosomes (Blumer-Schuette et al. 2014). Xylanosomes could be an extension of cellulosomes for an efficient degradation of lignocellulosic material in anaerobic bacteria such as *Acetivibrio*, *Ruminococcus*, and *Butyrivibrio* for the degradation of hemicelluloses (Doi et al. 2003; Lin and Thomson 1991). Xylanosome offers an enhanced breakdown of hemicellulose present in lignocellulosic biomass as compared to the xylanases of aerobes (McClendon et al. 2012). Zverlov et al. (2005) identified two new subunits of cellulosome in *C. thermocellum*, where one was xyloglucanase (Xgh74A), while another belongs to endoxylanase (Xyn10D). *Clostridium cellulovorans* possesses a cluster of xylanolytic enzymes with the molecular mass of 57 to 28 kDa. In an interesting study, varying expression of cellulosome's constituents has been observed in the presence of varying concentrations of xylan/cellulose substrates in the culture medium (Kosugi et al. 2012). Another strain of *C. thermocellum* was identified for GH-30 xylanases that showed activity toward glucuronic acid-free arabinoxylan (St. John et al. 2016). Recently, a thermostable acidic GH-10 endoxylanase (Xyn141E) was reported from *C. thermocellum* that showed optimal activity at pH 6.5 and 75 °C (Heinze et al. 2017). Very few of *Clostridium*-derived xylanases have been crystallized to date for characterization and, therefore, demand further research in this area.

Bacteroides belong to gram-negative, anaerobic rod-shaped bacteria that are abundantly reported to exhibit xylanase activity. Majority of such *Bacteroides* have been isolated from rumen and gut environments that include *Bacteroides eggerthii*, *Bacteroides ruminicola*, *Bacteroides intestinalis*, *Bacteroides xylanisolvens*, *Bacteroides cellulosilyticus*, *Bacteroides ovatus*, *Prevotella bryantii*, and *Bacteroides xylanisolvens* (Chassard et al. 2007; Dodd et al. 2010; Dodd et al. 2011; Zhang et al. 2014). *Bacteroides* plays a significant role in the degradation of organic compounds followed by their fermentation (Salyers 1995). *Bacteroides* exhibit xylanase activity on a broad range of xylan substrates and ability to ferment starch (Cooper et al. 1985). Majority of the *Bacteroides* like *B. ovatus* and *B. thetaiotaomicron* were detected as starch fermenters, where strain XB1AT of *B. xylanisolvens* is an exception (Chassard et al. 2007). Another strain of human gut, *Bacteroides intestinalis* DSM 17393, has been detected for two putative xylanases (Xyn8A and rex8A) of GH-8 family, where xyn8A was able to produce xylobioses and xylotrioses, while rex8A generated xyloses and xylobiose as the end products (Hong et al. 2014). Zhang et al. (2014) have studied extensively the profile of xylan-degrading enzymes of *Bacteroides* of the human gut.

Dictyoglomus, a distinguished taxon of thermophilic bacteria, has been characterized for thermostable xylanases (Gibbs et al. 1995; Zhang et al. 2010; McCarthy et al. 2000; Li et al. 2015a, b). In an earlier report, a thermostable xylanase has been identified from *Dictyoglomus* Rt46B.1 that showed optimum activity at 85 °C at slightly acidic pH of 6.5 (Gibbs et al. 1995). Zhang et al. (2010) overexpressed this xylanase along with the amylase gene in a heterologous host *Bacillus subtilis* WB800, where biophysical properties of the recombinant xylanase remained the same. On sequencing, it showed close homology with 16S rRNA sequences of

Dictyoglomus thermophilum. Several species of *Dictyoglomus* have been characterized for their unique xylanases to date. In another study, a strain *Dictyoglomus* sp. B1 revealed a xylanase active at 73 °C and pH 8.0 (Adamsen et al. 1995). The xylanase XYNB of *Dictyoglomus thermophilum* was stable at 80 °C and a high pressure of 500 MPa for 30 h (Li et al. 2015a, b). Low alkaliphilicity of *Dictyoglomus* xylanases limits their use in bleaching of pulp. *Thermoanaerobacter* (earlier identified as a member of *Clostridium*) belongs to the phylum *Firmicutes* which have been explored for their use in ethanol production from lignocellulosic biomass. The strains of *Thermoanaerobacter* are devoid of cellulase activity; therefore, they can be better targeted for their application in bleaching and de-inking of pulp and waste paper (Verbeke et al. 2013). A salt-tolerant xylanase of the GH-10 family was characterized from *Thermoanaerobacterium saccharolyticum* that exhibits activity at 70–73 °C at acidic pH (Hung et al. 2011). Three members of *Thermoanaerobacter* (*T. italicus* Ab9, *T. mathranii* subsp. mathranii A3, and *T. thermohydrosulfuricus* WC1) had been identified to possess a potent xylanase (Verbeke et al. 2013). Actinobacteria have also been reported to harbor xylanolytic enzymes. Xylanase of *Streptomyces* sp. QG-11-3 was characterized with optima 60 °C (Beg et al. 2000). A thermophilic actinomycete *Thermomonospora fusca* was identified to have a cluster of lignocellulose-hydrolyzing enzymes that include endoxylanase, α-L-arabinofuranosidase, β-xylosidase, and acetyl esterase (Bachmann and McCarthy 1991). Similarly, multiple xylanases have been detected in zymogram analysis from a novel species of *Streptomyces* that showed optimum activity at 70 °C (Brito-Cunha et al. 2013). *Streptomyces* sp. AOA40 produced a thermostable and salt-tolerant xylanase having a half-life of 172 min at 70 °C (Adiguzel and Tunçer 2016). At present, CAZy database records 1234 GH-10 and 234 GH-11 xylanases of bacterial type, where the contribution of *Micrococcus*, *Nocardia*, *Actinomadura*, and *Actinoplanes* cannot be ignored in accounting total number of available bacterial xylanases (Table 14.2). *Actinoplanes*, *Alkalitalea*, *Amphibacillus*, *Caldicellulosiruptor*, *Bacillus*, *Cellulomonas*, *Cellvibrio*, *Filimonas*, and *Granulicella* are the names of a few bacteria which produce xylanases from almost all the reported families to date.

14.11 Metagenomic Xylanases

Culture-dependent approaches have been exploited rigorously to obtain xylanases of interest. However, the majority of such xylanases do not fulfill the demand of the industries especially the paper/pulp and food/feed industries, where xylanases must be stable at higher temperatures and pH. Traditional culture-dependent microbial techniques lose 99.9% of microorganisms on Petri plates due to the limitations of nutrition and culture conditions (Verma and Satyanarayana 2011). Metagenomics offers an alternate and straight approach that deals with the cloning of community DNA and screening for different genes of interest by functional and sequence-based approaches. Xylanases have also been explored using the nontraditional advanced metagenomic strategies (Shi et al. 2011; Qian et al. 2015; DeCastro et al. 2016).

Table 14.2 Characteristics of various bacterial xylanases

Bacteria	Source	Opt. pH	Opt. $_{temp}$	MW (KDa)	GH family	Important features	Applications	References
Actinomadura sp. S14	Compost of Thailand	6.0	80.0	30–40 (with CBM)	Many GH families	Inhibition by Hg^{2+} and Cu^{2+}	Multiple xylanolytic activities	Sriyapai et al. (2011)
Caldicoprobacter algeriensis THC1(T)	Hydrothermal hot spring of Guelma (Algeria)	11.0	70	35.0	11	High xylobiose producer	Pulp bleaching industry	Amel et al. (2016)
Microcella alkaliphila JAM-AC0309	Deep subseafloor sediment cores, off Shimokita peninsula	8.0	65	150	10	$T_{1/2}$ of 48 hrs at 50 °C High molecular weight	Predominantly xylobiose production	Kuramochi et al. (2016)
Paenibacillus macerans IIPSP3	Termite gut	4.5	60.0	205.0	–	Half-life of 6 h at 60 °C and 2 h at 90 °C	Paper and pulp, biofuel	Dheeran et al. (2012)
Clostridium thermocellum	–	6.5	75.0	102.2	141	First report of GH-141 xylanase	XO production	Heinze et al. (2017)
Bacillus brevis	*Bacillus* genetic stock center (BGSC)	7.0	55.0	23.0	–	Cellulase-free	Paper and pulp industry	Goswami et al. (2014)
Bacillus amyloliquefaciens	Soil	7	80	18.5	–	Isoelectric point was 10.5 Complete inhibition by Hg^{2+}	–	Breccia et al. (1998)
Bacillus halodurans S7	Soda lake in the Ethiopian Rift Valley	9.0–9.5	75	43	–	Inhibited by Mn2+	–	Mamo et al. (2006)
Thermotoga thermarum	–	6.0	80.0	40.53	10	Retains 60% of its activity after 2 h at 75 °C	XO production	Shi et al. (2014)
Bacillus subtilis cho40	Chorao island of Mandovi estuary Goa, India	6.0	60.0	22.9	–	Halo tolerant	Proposed for bioethanol production	Khandeparker et al. (2011)

(continued)

Table 14.2 (continued)

Bacteria	Source	Opt. pH	Opt. temp	MW (KDa)	GH family	Important features	Applications	References
Glaceocola mesophila KMM 241	Marine environment	7.0	30.0	43.0	–	Cold active Retains 90% activity in 2.5 M NaCl	XO production	Guo et al. (2009)
Enterobacter sp.	Forest soil bacteria	6.0	40.0	61.66	43	1st xylosidase of *Enterobacter* Isoelectric point 5.7	–	Campos et al. (2014)
Caulobacter crescentus	Soil	6.0	45	63.0	43	Bifunctional enzyme		Graciano et al. (2012)
Streptomyces turgidiscabies C56	PCR	6.5	55.0	42.0	30	Acts on only side chain shaving GlcA/MeGlcA	Proposed for bio-energy	Maehara et al. (2018)
Amphibacillus xylanus	PCR	10.5	55.0	110.0	115	First GH-115 α-glucuronidase of the genus *Amphibacillus*	–	Yan et al. (2017)
Herbinix hemicellulosilytica	Thermophilic biogas reactor	6.0	55–60	58.8	10, 11 and 43	Exhibited cluster of xylanolytic enzymes	–	Mechelke et al. (2017)
Thermobifida halotolerans	PCR	9.0	70.0	34	–	Isoelectric point was 9.1 Xylotrioses as an end product	Proposed as food source	Zhang et al. (2012)
Paenibacillus campinasensis	PCR	7.0	60.0	41.0	11	K_M of 6.78 mg/ml and a Vmax of 4953 Mol/min/mg	–	Ko et al. (2010)
Sulfolobus solfataricus	–	7.0	90.0	57.0	11	Half-life of 47 min at 100 °C Weak Avicel activity	–	Cannio et al. (2004)
Caldicellulosiruptor bescii	PCR	7.2	70.0	40.0	10	Xylobiose was the predominant product Half-life of about 7.7 h at 60 °C	XO production	An et al. (2015)
Bacillus pumilus B20	Paper mill soil sample	6.5	60.0	85.0	–	High XO production	Prebiotic XO syrup	Geetha and Gunasekaran (2017)

Caldicellulosiruptor owensensis		7.0	90.0	43.0	10	Half-life of about 1 h at 80 °C	–	Liu et al. (2018)
Acinetobacter pittii MASK25	Soil	5.0	40.0	–	–	Predominantly xylopentaose and xylohexaose producer	Agro-residue saccharification	Purohit et al. (2017)
Streptomyces coelicolor A3(2)	Soil	6.0	60.0	47.0	10	K_M of 0.24 mg/mL and Vmax of 6.86 μM/min	Specifically releases xylobiose and xylopentaose	Enkhbaatar et al. (2016)
Lactobacillus rossiae DSM 15814^T	Sourdough	6.0	40.0	–	43	Hydrolyzed glycosidic linkages from xylan and/or arabinoxylan liberating XOs	Xylose producer	Pontonio et al. (2016)
Saccharophagus degradans 2–40	Marine environment	7.0	30.0	–	10	Cold active; K_M 10.4 mg mL^{-1}; Vmax = 253 μmol mg^{-1} min^{-1}	XO production	Ko et al. (2016)
Geobacillus thermodenitrificans A333	–	7.5	70.0	44.0	–	Salt tolerant up to 3.0 M NaCl	Xylobiose and xylotriose as main products	Marcolongo et al. (2015)
Streptomyces mexicanus HY-14	Insect symbiont	5.5	65.0	44.0	10	Xylobiose (>75%) as the primary degradation product	XO production	Kim et al. (2014a, b)
Caldicellulosiruptor lactoaceticus 6A	PCR	6.5	80.0	47.0	10	90% residual activity after incubation at 75 °C for 6 h	XO production	Jia et al. (2014)
Streptomyces sp. AOA40		6.0	60	–	–	Half-life of 172 min at 70 °C	Bread making; juice clarification	Adiguzel and Tunçer (2016)

Approximately 200 xylanase-encoding genes have been retrieved successfully to date, where most of them are not well characterized. It is important to mention here that most of the retrieved xylanases fall into the GH-10 family based on high molecular weight and low isoelectric point. The hot pool of New Zealand revealed the first metagenomic xylanase using genome-walking PCR (GWPCR). On characterization, it showed an optimum activity at 100 °C under acidic conditions (Sunna and Bergquist 2003). This was the first report to access the xylanase gene using the degenerate sets of primers. Thereafter several microbial xylanase-encoding genes have been fished out using the signature sequences of xylanases (Helianti 2007; Hayashi et al. 2005; Liu et al. 2005; Sheng et al. 2015). Amplification of genes using degenerate sets of primers from a metagenome enhances the possibilities to get the novel xylanase sequences as compared to the use of specific primers, where known sequences are used for designing primers (Verma et al. 2010; Sheng et al. 2015). Gut microbiota of different organisms could be a rich source of microbes for exploring the genes of varied interests. Intestinal microflora of herbivores could be a reservoir of lignocellulose-degrading genes (Leth et al. 2018). Several potential and diverse xylanases have been reported using the metagenome of the gut microbiota (Sheng et al. 2015; Leth et al. 2018). Novel endoglucanase, β-glucosidase, and cellulodextrinase have been identified from the cosmid-based metagenomic library of buffalo rumen (Liu et al. 2009; Cheng et al. 2012). The human gut is almost untouched for retrieving xylanase-encoding genes using nontraditional metagenomic approaches. Five GH-10 family xylanases were discovered from the human gut that showed their similarity with xylanases of anaerobic *Bacteroidetes*. Of which, one xylanase was extensively characterized that displayed optimal activity at 40 °C at pH 6.5 (Hayashi et al. 2005). A broad spectrum transcriptomic analysis of the human gut bacteria revealed that dietary fibers enhance the expression of GH-10 xylanases, which is a conserved GH family of the phylum *Bacteroidetes* (Krishnan et al. 2014). The gut of insects is another very interesting reservoir for lignocellulose degraders (Brennan et al. 2004; Shi et al. 2013; Krishnan et al. 2014). Order Lepidoptera, Isoptera, Orthoptera, and Coleoptera have been used for exploring xylanases of varying properties. In an interesting report, three GH-11 and one GH-8 acidic xylanases were discovered from the insect gut metagenome that showed the substrate specificity toward arabinoxylan (Brennan et al. 2004). Comparative analysis of the gut microbiota of various orders of the class Insecta revealed that grasshoppers (Orthoptera) and woodborers (Coleoptera) possess better xylanase activities than the leaf-consuming insects of Lepidoptera (Shi et al. 2011). In addition, the study concluded that Coleoptera can be further explored for alkaline xylanases. In another investigation, gut metagenome of termite and horse were enriched using rolling circle amplification to enhance the copy number of target DNA. Thus, the pre-amplified DNA was used to amplify the xylanase-encoding genes using inverse PCR that revealed two putative xylanases of GH-10 family from vermiform appendix of a horse (Yamada et al. 2008). Another report from termite gut metagenome revealed a GH-11 xylanase (xyl7) that showed enzymatic activity in a broad pH range of 5.5–10.0 (Qian et al. 2015). Sheng et al. (2015) retrieved a plethora of xylanase genes of GH-10 (19 genes), GH-11 (14 genes), and GH-8 (27 genes) families

using degenerate set of primers from the gut metagenome of *Holotrichia parallela* larvae. Thermostability of xyl7 was improved by 250-fold at 55 °C along with an increase in optimum temperature by 10 °C using directed evolution approach (Qian et al. 2015). One BAC-based library from the cattle rumen metagenome revealed an open reading frame of xylanase out of the 68 ORFs. Its subsequent cloning, expression, purification, and characterization showed that the xylanases display optimal activity at 50 °C at pH 6.0 (Zhao et al. 2010). Environmental samples like soil, compost, water, effluents, and wastes have been explored extensively as compared to any other source(s) (Wang et al. 2010; Leth et al. 2018; Basit et al. 2018). A cold-active and high molecular weight (~46 kDa) xylanase of family GH-8 had been retrieved from environmental metagenomic library. Xylanases of GH-8 are known for their applications in pharmaceutical industry having narrow substrate spectrum with liberation of specific xylooligosaccharides (Lee et al. 2006). GH-8 xylanase is one of the less explored xylanases by both cultivation and metagenomic approaches that calls for further investigation. Cold-active xylanases have been retrieved from a soil-based metagenomic library, which were categorized into GH-10 family. Further, they were slightly alkaline having sustainable activity in the presence of various divalent metal ions (5 mM), an industrially relevant property (Hu et al. 2008). Conversely, a xylanase obtained from manure metagenomic library was totally inhibited in the presence of 10 mM Cu^{2+} (Li et al. 2009). Alpine tundra soil had also been mined for xylan degraders. Wang et al. (2010) successfully retrieved several cold-active xylanase-encoding genes of families 10 and 11 from an environmental metagenome of alpine tundra soil using degenerate sets of GH-10 and GH-11 primers (Wang et al. 2010). These xylanases displayed very little similarity with the available GH-10/GH-11 xylanase sequences available in GenBank database; moreover, their extensive characterization has also not been reported till date (Wang et al. 2010). Compost soil grabbed attention due to its complex microbial diversity. Metagenomics has also been employed to dig out the xylanases of interest from compost soil by several investigators (Kwon et al. 2010; Jeong et al. 2012; Verma et al. 2013a). It is a well-known fact that environment impacts inhabitant microbes and their proteins/enzymes. Compost soil is known for its acidic nature; Kwon et al. (2010). Jeong et al. (2012) found acidic xylanases from compost soil-based metagenomic libraries. Kwon et al. (2010) retrieved five xylanase-positive clones along with several cellulase-encoding genes from the metagenomic library. All five xylanases showed optima at 50 °C except the xylanase of clone X5 that exhibited optimum activity at of 55 °C. As expected, these xylanases were acidic with activity in the pH range of 5.5 to near 7.0 (Kwon et al. 2010). Xylanases with almost similar properties (temp opt = 40 °C and pH_{opt} = 6.0) of xylanases (Xyn10J) were reported by Jeong et al. (2012), where fosmid-based compost metagenomic library was screened (Jeong et al. 2012). Xylanase-encoding gene (1137 bp) from the recombinant clone was fished out and sub-cloned into cloning vector pUC19 along with its native signal peptide. Sequence analysis showed maximum identity (60%) with the xylanases of an uncultured bacterium *Cellvibrio mixtus*. Another interesting and unusual xylanase discovered from compost soil metagenome showed optimum activity at 80 °C and pH 9.0 with $T_{1/2}$ of 2 h at 80 °C. It was quite unusual in

properties due to its retrieval from an acidic habitat of compost. This was the first GH-11 xylanase retrieved by metagenomic approach with alkalistability and thermostability (Verma et al. 2013a). This xylanase suits well for paper and pulp industry. Metagenomic library from chicken's cecum metagenome yielded a xylanase-positive clone; the enzyme displayed optimum activity at 45 °C and pH 6.0. The purified GH-10 xylanase was salt tolerant and retained 72% activity at 60 °C (Al-Darkazali et al. 2017). Holstein cattle rumen revealed occurrence of a novel GH-10 xylanase (Xyln-SH1) that showed 44% identity with the glycosyl hydrolase from *Clostridium thermocellum* ATCC27405. On characterization, the cellulose-free Xyln-SH1 exhibited optimal activity at pH 6.0 and 40 °C and high specificity toward softwood xylans. Another very similar GH-10 xylanase (XynH) had been discovered from a soil-based metagenomic library that exhibited optimum activity at 40 °C and pH 7.2. BLAST analysis revealed that the XynH has the maximum identity of 56% with XylC of *Cellvibrio mixtus* (GenBank accession number AF049493) (Hu et al. 2008). Mori et al. (2014) had also reported a xylanase-encoding gene from soil metagenome. The raw soil was repeatedly enriched using cellulose to enhance the count of lignocellulose degraders followed by construction of soil metagenomic library and screening for cellulase- and xylanase-positive clones (Mori et al. 2014). The CAZy database reflects the increasing figures of xylanases from an uncultured source that comprises 301 of GH-10 and 107 of GH-11 xylanase proteins.

14.11.1 β-Xylosidases

β-Xylosidase (exo-1,4-β-D-xylosidase EC 3.2.1.37) liberates xylose as an end product by cleaving β-1-4-glycosidic linkages in short xylooligosaccharides and xylobioses from the nonreducing end. True β-xylosidases can hydrolyze the artificial substrates like para-nitrophenyl β-D xylopyranoside (pNPX) to liberate p-nitrophenol (Coughlan and Hazlewood 1993). The affinity of β-xylosidases decreases with increase in the degree of polymerization of oligosaccharides. The majority of the β-xylosidases are cell-associated, which act on hydrolyzed products of xylanases. Despite the availability of several β-xylosidases, culture-independent approaches have also been employed for discovering novel β-xylosidases especially with thermo- and alkalistabilities and salt tolerance (Zhou et al. 2012; Bastien et al. 2013). With best efforts, only handful of β-xylosidases has been reported till now using metagenomic approaches. In a pioneering report, five β-xylosidases have been reported from the yak rumen metagenome (Zhou et al. 2012). The bifunctional xylanases (RuBGX1) showed dual properties having simultaneous saccharification of cellulose and xylan for releasing the glucose and xylose simultaneously. A broad range of substrates like p-nitrophenyl-L-arabinofuranoside (pNPA), nitrophenyl-β-D-xylopyranoside (pNPX), and xylooligosaccharides have been used in enzyme assays (Zhou et al. 2012), which has pharmaceutical application due to transxylosylation activity. Ferrer et al. (2012) reported 15 glycosyl hydrolases from the cow rumen metagenome, where an interesting multifunctional enzyme (R_09–02)

exhibited β-1,4-xylosidase, α-1,5-arabinofur(pyr)anosidase, β-1,4-lactase, α-1,6-raffinase, α-1,6-stachyase, β-galactosidase, and α-1,4-glucosidase activities. The retrieved gene showed identity with GH-53 family of Clostridia (Ferrer et al. 2012). Shotgun sequencing of switchgrass-adapted compost metagenome resulted in 22 putative ORFs, where 4 ORFs were predicted for β-xylosidase activity along with the stretches of xylanases, acetyl xylan esterase, and cellulase. Two of them were successfully cloned, expressed, and characterized (Dougherty et al. 2012). Two of the ORFs (JMC25406 and JMC04168) were successfully expressed in soluble form having bifunctional β-xylosidases/α-arabinofuranosidase activities at pH 5.5. Sequence analysis categorized these moderately thermostable β-xylosidase-encoding ORFs (JMC25406, JMC04168, and JMC35591) into GH-43 family, while the fourth one (JMC44805) shared the identity with the sequences of GH-39 family (Dougherty et al. 2012). Several β-D-xylosidase- and α-L-arabinofuranosidase-positive clones were harnessed from the gut of the termite *Pseudacanthotermes militaris* (Bastien et al. 2013). A total of 82 thermostable β-xylosidases were successfully expressed out of the 269 proteins. Majority of these industrially relevant β-xylosidases showed optimal activity at 50 °C, while few of them were able to retain activity even at 90 °C (Sato et al. 2017).

14.12 Biotechnological Applications of Xylanases

Microbial enzymes offer an eco-friendly, cost-effective, and sustainable alternative to harmful chemicals used in various industries during downstream processing of raw materials to value-added products. Enzymes have been deployed successfully in various biotech as well as non-biotech industries (Fig. 14.2). According to the survey of BCC Research, a global market of microbial enzymes is expected to increase to $6.3 billion by 2021 at a compound annual growth rate (CAGR) of 4.7% for 2016–2021 (www.bccresearch.com). Xylanases share a major chunk of industrially relevant enzymes because of their potential and widespread applications (Verma and Satyanarayana 2016; Basit et al. 2018). Moreover, bacterial xylanases have a special attention in industries due to the availability of broad-spectrum xylanases, their cellulase-free nature, and their ability to withstand the harsh physical conditions of the industries. Over the past decade, global market of xylanases has shot up significantly worldwide (Singh et al. 2016). Due to the wide bioprospects of microbial xylanases, the following are the major areas that must be extensively discussed for their potential applications.

14.12.1 Paper and Pulp Industry

Xylanases attracted attention of paper and pulp industries in 1986, when Viikari et al. (1986) reported their applicability in pulp bleaching. Thereafter, the demand for thermoalkalistable xylanases increased significantly. The kraft cooking process of chopped woods occurs at a high temperature of 170 °C under alkaline conditions

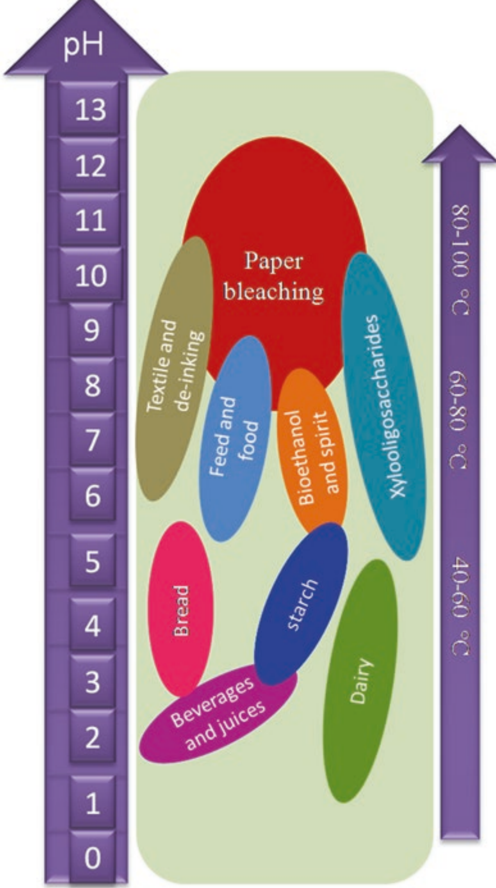

Fig. 14.2 Temperature and pH ranges of xylanolytic enzymes and their applications in various industries

to soften the chips. This step makes the pulp hot and alkaline; after the first washing, temperature and pH go down and come in the range of 90–110 °C and pH 9.0–11.0. Bleaching of pulp occurs at this stage by using the chemicals like chlorine/chlorine dioxide. Therefore, xylanases must be thermoalkalistable to cope with the conditions. Xylanases have been reported to replace the chlorine by 20–35% (Verma and Satyanarayana 2013d; Kumar et al. 2016; Sato et al. 2017). Moreover, it also reduces the formation of dioxins, which are generated during a chemical reaction between lignin and chlorine. Dioxins are neurotoxic, mutagenic, and persistent in nature (Sharma et al. 2007; Dutta et al., 2017). Although several xylanases have been reported till date, majority of them do not meet the needs of paper and pulp industries. A handful of bacterial xylanases are available that exhibit dual properties of thermostability and alkalistability at extreme pH and temperatures which were successfully employed in bleaching of the pulp from various sources (Manimaran et al. 2009; Khandeparkar and Bhosle 2007; Garg et al. 2011; Lin et al. 2013a, b; Verma and Satyanarayana 2013d; Kumar and Satyanarayan 2012;

Cheng et al. 2013; Fillat et al. 2010). Metagenomic xylanase (Mxyl) from compost soil metagenome has been successfully utilized to make the paper white and bright (Verma and Satyanarayana 2013d). This led to a reduction of 29% in chlorine consumption due to the recombinant Mxyl enzyme. A thermostable xylanase from a polyextremophilic *B. halodurans* TSEV1 enhanced brightness with 14.7% reduction in kappa number (Kumar and Satyanarayana 2011). Another thermophilic and basophilic xylanase of *B. halodurans* C-125 showed the bleach boosted effect on the pulp of wheat straw by a significant release of chromophores and hydrophobic compounds in the filtrate (Lin et al. 2013a, b). Xylanase from *Geobacillus thermodenitrificans* TSAA1 also showed the significant release of chromophores from pulp samples (Verma et al. 2013b). Similarly, xylanase of *Streptomyces griseorubens* LH-3 reduced the kappa number by 15.3% with a significant increase in brightness (Cheng et al. 2014).

14.12.2 Food Industry

Thermostable xylanases find application in food industry, especially in bread and baking industries (Harris and Ramalingam 2010). Xylanases have been reported to play a role in increasing the dough size of the bread and to make it soft and puffy (Subramaniyan and Prema 2002). Cocktail of amylase and xylanase enhances the flavor of bread by hydrolyzing starch and xylan to release sugars (Mandal 2015; Kumar et al. 2017). Moreover, xylanases incorporate xylooligosaccharides (XOs) and arabino-xylooligosaccharides into the bread during the bread processing. XOs act as prebiotics by augmenting the growth of several beneficial gut microorganisms like *Bifidobacterium bifidum*, *Bacteroides fragilis*, and several *Enterococcus* species (Aachary and Prapulla 2011; Verma and Satyanarayana 2012b). XOs have also been reported to inhibit the proliferation of harmful pathogens present in the gastrointestinal tract (Mandal 2015; Jain et al. 2015; Kumar et al. 2017). Alkalistable and thermostable xylanases from *Streptomyces thermocyaneoviolaceus*, *G. thermoleovorans*, and *B. halodurans* have been successfully used in generating XOs from wheat bran (Shin et al. 2009; Kumar and Satyanarayana 2011; Verma and Satyanarayana 2012b). Awalgaonkar et al. (2015) have successfully used xylanases in making papad, an Indian traditional snack made of various pulses and cereals. Incorporation of xylanases into the black gram based dough of papad that significantly improved its quality (Awalgaonkar et al. 2015). Xylanase has been employed in biscuit industry for making the cream crackers lighter, nutritive, and palatable with improved texture (Polizeli et al. 2005).

14.12.3 Feed Industry

Animal feed is broadly categorized into two groups: fodder and forage. Fodder includes pelleted feeds, silage, straw, hay, sprouted grains, legumes, and seeds, while forage is chiefly a plant material like leaves and stems (Benedict et al., 2017;

Soder et al. 2018). Therefore, feedstuff is rich in lignocellulosic ingredients and demands xylanases for facilitating the release of entrapped sugars/nutrients within. Xylanase mixed feed enhances the nutrient value of the feed by hydrolyzing the lignocellulosic material and releasing the sugars and xylooligosaccharides. Stachyose and raffinose like non-starch-based polysaccharides (NSP) are non-available sugars for animals; therefore xylanase treatment degrades the NSP complex and releases nutrients making them available for animals. Moreover, the presence of xylanases enhances further in monogastric animals where xylanolytic microflora is absent, therefore demanding xylanase-treated chunks for proper digestion (Verma and Satyanarayana 2012a). A commercial enzyme mix of Avizyme (xylanases, amylase, and protease) has been successfully used in a soy-based diet in poultry industries (Cafe et al. 2002). Similarly, xylanase-treated diet showed the enhanced production and improved quality of eggs in egg-laying quail (Bayram et al. 2008).

14.12.4 Lignocellulose Bioconversions

Saccharification of lignocellulosic materials from agro-based residues grabbed attention by pharmaceutical industries. Xylanases facilitate the release of various sugars and xylooligosaccharides (XOs) from lignocellulosic residues, which find medical applications. Xylooligosaccharides have been proven for their role in preventing dental caries (Verma and Satyanarayana 2012a). XOs have beneficial health effects that improve the growth of healthy microbiota of the gut (Yang et al. 2015). In addition, sugars and XOs can be further transformed into other products of human interest like ethanol, hydrogen, and organic acids (Barnard et al. 2010; Lo et al. 2010; Li et al. 2011). Wheat bran, corn cobs, and sugarcane bagasse have been used in the production of XOs by employing xylanases of metagenomic origin and recombinant xylanase of *G. thermodenitrificans* TSAA1 (Verma et al. 2013a; Verma et al. 2013b). XOs with inulin ameliorate immune status and intestinal environment (Lecerf et al. 2012; Li et al. 2015).

14.12.5 Preparation of Juice from Fruits and Vegetables

Fruit and vegetable-based juice industries demand a combination of enzymes for extracting pulp from the fruits, their liquefaction, and clarification. Microbial enzymes like cellulase, pectinase, and amylase along with xylanases assist in this multistep process. Xylanases in conjunction with other enzymes stabilize the pulp, improve the yield, increase aroma, and reduce viscosity (Polizeli et al. 2005). The fruits and vegetables are also used in the recovery of essential oils, flavoring agents, vitamins, pigments, edible dyes, and mineral salts, where xylanases facilitate recovery of products of interest (Polizelli et al. 2005; Mandal 2015). There are several reports on the successful use of xylanases alone as well as in combination with other enzymes for attaining high yield of fruit and vegetable juices (Dhiman et al. 2008;

Pal and Khanum 2011). Nagar et al. (2010) used an acid-stable xylanase from *Bacillus pumilus* SV-85S for enzymatic clarification of juices from apple (*Malus domestica*), pineapple (*Ananas comosus* L.), and tomato (*Lycopersicum esculentum*) (Nagar et al. 2010). In addition, incorporation of xylanase further improved the yield by 10–25%. In another interesting study, immobilized xylanases on 1,3,5-triazine-functionalized silica-encapsulated magnetic nanoparticles led to significant clarification of fruit juice (Shahrestani et al. 2016).

14.13 Waste Paper De-Inking and Textile Industry

Recycling of waste paper is a chief concern of environmental management. Due to the environmental initiatives and economic prospects, recycling of waste paper is in high demand. De-inking of printed papers is one of the crucial steps during the recycling process of the used papers that uses hazardous chemicals. Consequently, a huge amount of derived chemical moieties releases into the environment and affects the living organisms and their surroundings. Combination of microbial enzymes has been employed successfully for de-inking the waste paper (Lee et al. 2013; Virk et al. 2013; Kumar and Satyanarayana 2014). Xylanase, laccase, and ligninase play a significant role in paper de-inking. Bacterial xylanases have been preferred because of their cellulase-free nature. Singh et al. (2012) reported for the first time the use of bacterial xylanases in de-inking of school-generated waste paper, where 15 IU of xylanases and 30 IU of pectinase significantly lowered BOD and COD values by 20.15% and 22.64%, respectively (Singh et al. 2012). A thermoalkalistable and cellulase-free xylanase of *B. halodurans* FNP 135 was successfully employed to de-ink the newsprint along with alkaliphilic laccase (Virk et al. 2013). Another strain of *B. halodurans* TSEV1 has also been used in removing the ink from the printed papers (Kumar and Satyanarayana 2014). Combination of commercial cellulase (1.2 U/mg) along with thermoalkaliphilic xylanases (1.4 U/mg) showed maximum de-inking during the process at 70 °C (Kumar and Satyanarayana 2014). Surprisingly, a strain of *Escherichia coli* SD5 had been reported to possess lignocellulosic activity for the first time (Kumar et al. 2018). The produced xylanase caused a significant reduction in kappa number during de-inking of the waste paper.

14.13.1 Oil Seed Extraction

Due to the presence of significant amount of lignocellulosic material in the oilseeds, xylanases and cellulases can be used to facilitate easy extraction of oils (Rosenthal et al. 1996). Xylanases have been reported to enhance the yield of oil during their extraction from different seeds (Marasabessy et al. 2011). Xylanase-producing strain of *Bacillus pumilus* and *Bacillus altitudinis* MB4 assisted in enhanced oil production from *Jatropha* seeds (Marasabessy et al. 2011). A commercial xylanase has been used to digest the palm oil mill effluent for biogas production (Prasertsan et al. 2017).

14.13.2 Bioethanol Production

As there is a competition for the first-generation crops as a food source, the idea of using the crop residues as raw material for the bioethanol production seems feasible. Second-generation energy crop/lignocellulosic biomass is an attractive alternative for the production of the bioethanol as compared to the first-generation crops in a cost-effective manner. Moreover, lignocellulosics as fuel can assist in reducing the emission of greenhouse gases (Ballesteros et al. 2006). Lignocellulosics are abundant in hexose and pentose sugars which can be fermented to bioethanol (Ire et al. 2016). However, these sugars are entrapped in the complex structural composition of lignocellulosics and need to be released to make them available for fermentation. Cellulases and xylanases from bacterial sources have always been a choice for bioethanol production due to their broad substrate range and higher operating temperatures (Scully and Orlygsson 2015). More than 300 strains of bacteria have been reported to be useful in bioethanol production by 2008, where the USA and Brazil account for major chunk of 85% of total bioethanol production worldwide (Wagner and Wiegel 2008; Bertrand and Vandenberghe 2016). Ethanologenic thermophilic bacteria include the species of genera *Caldanaerobacter*, *Thermoanaerobacterium*, and *Clostridium* (Taylor et al. 2006; Tomas 2013). The members of thermophilic bacteria *Bacillus*, *Geobacillus*, *Caloramator*, and *Paenibacillus* have also shown been reported for bio-ethanol conversion (Sveinsdottir et al. 2009; Tomas 2013). Xylanase, β-xylosidase, and cellulases are the main enzymes deployed in generating fermentable sugars from lignocellulosic biomass their fermentation to into ethanol. Xylanase along with β-xylosidase acts on xylan and xylooligosaccharide for releasing xylose sugars (Jain et al. 2014). Additionally, xylanases loosen the cellulosic fibers for enhancing their accessibility to cellulase, which produces fermentable sugar glucose. The action of cellulases and xylanase generated 0.567 g/g of sugars, which yielded 0.172 g bioethanol/g biomass after 24 h of fermentation (Wi et al. 2013). Simultaneous saccharification and co-fermentation (SSCF) of *Bacillus cereus* GBPS9 and *Bacillus thuringiensis* resulted in bioethanol production from sugarcane bagasse (Ire et al. 2016). Co-culturing of bacteria has been reported for high yield of bioethanol as compared to single-cell biotransformation (Wi et al. 2013; Ire et al. 2016). *Clostridium thermocellum*, an anaerobic thermophilic bacterium, is unable to ferment C_5 sugars; therefore co-culturing of *C. thermocellum* with ethanologenic bacteria like *Thermoanaerobacterium* and *Thermoanaerobacter* can produce bioethanol efficiently (Demain et al. 2005). An engineered *C. thermocellum* yielded 38.1 g/l ethanol from crystalline cellulose, when co-cultivated with *Thermoanaerobacterium saccharolyticum*. This is the highest yield of ethanol production reported from any cellulolytic co-cultivation approach to date (Argyros et al. 2011).

14.14 Conclusions and Future Perspectives

Microorganisms are well known for their role in degrading xylan and other hemicelluloses of plant organic matter by secreting several xylanolytic and hemicellulolytic enzymes. Although fungal xylanases exhibit high enzyme activity, their instability at elevated temperatures limits their application in various industrial processes. Bacterial xylanases offer several advantages over the other xylanases, which include their optimum enzymatic activity in a broad pH and temperature ranges, cellulase-free nature, low molecular weight, broad substrate specificity, and high stabilities at elevated temperatures and in alkaline conditions. Easiness in cultivation, rapid growth, and fast recovery of the enzyme and availability of DNA manipulation methods make bacteria the microbes of choice for producing xylanases. Members of *Actinomadura*, *Thermotoga*, *Geobacillus*, *Dictyoglomus*, and *Clostridium* are some of the bacteria of choice, which produce xylanases with stability at elevated temperatures and high pH. Due to the heterogeneity in xylan substrate, multiplicity in xylanases occurs, and these have been categorized into 20 glycosyl hydrolase families. The CAZy database classifies these xylanases based on amino acid sequence similarity, where GH-10 and GH-11 xylanases are the most significant glycosyl hydrolases because of their direct attack on the backbone of the xylan. Both these xylanases display retention mechanism of catalysis having glutamate as nucleophile/base as well as a proton donor. GH-11 xylanases are considered as true xylanases because of their high specificity toward the substrate. Archaeal xylanases also exhibit tolerance to temperatures as high as 100–105 °C. Archaeal xylanases are acidic in nature that limits their wider applications in the industries. DNA shuffling, directed evolution, and site-directed mutagenesis are the techniques which were employed successfully for improving the desirable properties of existing xylanases. The culture-independent metagenomic approaches aid in retrieving xylanases with novel properties from various environmental sources. Gene sequences encoding 20 families of xylanolytic enzymes have been successfully identified. Majority of them are still uncharacterized and grouped into unclassified category; therefore, extensive efforts are called for their characterization. Bacterial xylanases are being applied in various industrial processes, where MEGAZYME (https://www.megazyme.com), PROZOMIX (http://www.prozomix.com), and NZYTech (https://www.nzytech.com) are the names of a few organizations that are commercializing xylanases. *Bacillus stearothermophilus* T-6, *Cellvibrio japonicus*, *C. mixtus*, and *Thermotoga maritima* are the bacterial strains that are being used in the production of GH-10 xylanases. Most of the commercial GH-11 xylanases are of fungal origin. Further efforts are needed to search for GH-11 xylanases from prokaryotic bacteria and archaea and through environmental metagenomes. DNA manipulations, therefore, can be an alternative approach for modifying the existing xylanases to make them functional in extreme conditions of industrial processes. Culture-dependent and culture-independent metagenomic techniques may be employed in finding ideal xylanolytic enzymes.

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15 Diversity in Cellulose-Degrading Microbes and Their Cellulases: Role in Ecosystem Sustainability and Potential Applications

Anica Dadwal, Shilpa Sharma, and Tulasi Satyanarayana

Abstract

Cellulose is a renewable carbon source, which is an abundant natural biopolymer present in agricultural and forest residues. A great variety of prokaryotic and eukaryotic microbes are known to produce cellulose-degrading enzymes. Besides well-known cellobiohydrolase, endoglucanase and β-xylosidase, other enzymes such as lytic polysaccharide monooxygenase (LPMO) have been reported to play a role in cellulose hydrolysis. This chapter focuses on the diversity of cellulose-degrading microbes and various cellulolytic enzymes produced by them. Cellulases are one of the widely used enzymes in textile, paper and pulp, wine and brewery, biofuel and pharmaceutical industries. The role of cellulose degradation in the ecosystem sustainability and multifarious biotechnological applications of cellulases are briefly described.

Keywords

Biofuel · Bioethanol · Lignocellulosics · Cellulases · Cellulose-degrading microbes · Ecosystem sustainability

15.1 Introduction

Cellulose is an abundantly available biopolymer made of glucose units linked by β-(1,4) linkages. The hydrolysis or breakdown of cellulose requires a synergistic action of enzymes known as cellulases, which are placed in the glycoside hydrolase (GH) family of enzymes and act by cleaving β-1,4-glycosidic bonds in

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polysaccharides and oligosaccharides. Cellulolytic enzymes consist of three major components: exoglucanases, endoglucanases and β-glucosidases. Endoglucanases cleave internal bonds of cellulose, while exoglucanases cleave small polysaccharide units from the ends of the exposed chains into disaccharides yielding cellobiose. β-Glucosidases then hydrolyse cellobiose and cello-oligosaccharides into individual monomeric units, glucose. These three enzymes act in concert to completely hydrolyse cellulose. Due to their ability to convert cellulosic materials into useful sugars, these enzyme systems have attracted attention in search of alternative ways to meet energy demands. Thus, cellulases have been considered to play an important role in biofuel production. Various microbes have been widely explored in producing a wide variety of cellulases. Many cellulolytic bacteria and fungi have been isolated from diverse habitats such as composting heaps or municipal solid waste (MSW), decaying plant materials, mangroves, gut microbiota of ruminants, soil and hot springs. Diverse habitats have been the source of several different kinds of cellulases, which differ in their amino acid sequences and structures. Various cellulases have been surveyed in order to get an efficient microbe that produces cellulases with high cellulose saccharifying potential, which could be exploited under industrial processing conditions. This chapter focuses on the diversity of cellulose-degrading microbes and their cellulases. Research efforts made in characterizing cellulases from different microbial sources have been discussed. Cellulases play a role in ecosystem sustainability by their role in carbon cycling, symbiotic relations between invertebrates and microbes, symbiotic root hair infection of legumes, protection of plants against pathogens and various industrial processes. Multifarious applications of cellulases are also discussed.

15.2 Diversity in Cellulose-Degrading Microbes

Microorganisms are the key sources of enzymes that decompose cellulose and hemicelluloses present in the cell walls of plant biomass. It is assumed that fungi are the major source of these enzymes, although numerous bacterial strains are also known to degrade cellulose. Cellulase-producing microbes are listed in Table 15.1. Most of the cellulase-producing strains belong to the phyla *Firmicutes*, *Actinobacteria*, *Proteobacteria* and *Bacteroidetes* (Ulrich et al. 2008). Various hubs for cellulose-degrading enzymes are known in nature. One of the well-studied sources is organic municipal solid waste (MSW), which is rich in cellulosic components. Sarsaiya et al. (2017) reported isolation of four fungi *Trichoderma viride*, *Aspergillus niger*, *Chaetomium globosum* and *Fusarium oxysporum* from MSW. Among these, the highest enzyme activity was reported in *Trichoderma viride* and *Aspergillus niger*. Cellulolytic bacteria were isolated from rice straw waste and MSW from different areas of Sylhet district, Bangladesh. They were identified as *Bacillus* spp., *Pseudomonas* sp. and *Serratia* sp. based on biochemical tests and morphological and cultural characteristics. Maximum level of cellulase production was reported by *Bacillus* and *Serratia* followed by *Pseudomonas* isolates (Khatiwada et al. 2016). From the sample collected from MSW dumped in

Table 15.1 Cellulase-producing microbes

Group	Classification	Species
Bacteria	Aerobic bacteria	*Acinetobacter junii*, *A. amitratus*, *Acidothermus cellulolyticus*, *Anoxybacillus* sp., *Bacillus subtilis*, *B. pumilus*, *B. amyloliquefaciens*, *B. licheniformis*, *B. circulan*, *B. flexus*, *Bacteroides* sp., *Cellulomonas biazotea*, *Cellvibrio gilvus*, *Eubacterium cellulosolvens*, *Geobacillus* sp., *Microbispora bispora*, *Paenibacillus curdlanolyticus*, *Pseudomonas cellulosa*, *Salinivibrio* sp., *Rhodothermus marinus*
	Anaerobic bacteria	*Acetivibrio cellulolyticus*, *Butyrivibrio fibrisolvens*, *Clostridium thermocellum*, *C. cellulolyticum*, *C. acetobutylium*, *C. papyrosolvens*, *Fibrobacter succinogenes*, *Ruminococcus albus*
Fungi	Soft rot fungi	*Aspergillus niger*, *A. nidulans*, *A. oryzae*, *A. terreus*, *Fusarium solani*, *F. oxysporum*, *Humicola insolens*, *H. grisea*, *Melanocarpus albomyces*, *Penicillium brasilianum*, *P. occitanis*, *P. decumbans*, *Trichoderma reesei*, *T. longibrachiatum*, *T. harzianum*, *Chaetomium cellulyticum*, *C. thermophilum*, *Neurospora crassa*, *P. fumigosum*, *Thermoascus aurantiacus*, *Mucor circinelloides*, *P. janthinellum*, *Paecilomyces inflatus*, *P. echinulatum*, *Trichoderma atroviride*
	Brown rot fungi	*Coniophora puteana*, *Lanzites trabeum*, *Poria placenta*, *Tyromyces palustris*, *Fomitopsis* sp.
	White rot fungi	*Phanerochaete chrysosporium*, *Sporotrichum thermophile*, *Trametes versicolor*, *Agaricus arvensis*, *Pleurotus ostreatus*, *Phlebia gigantea*
Actinobacteria		*Cellulomonas fimi*, *C. bioazotea*, *C. uda*, *Streptomyces drozdowiczii*, *S. lividans*, *Thermomonospora fusca*, *T. curvata*
Protozoa		*Epidinium caudatum*, *Pseudotrichonympha grassii*, *Eudiplodinium maggii*, *Ostracodinium obtusum bilobum*, *Polyplastron multivesiculatum*, *Entodinium* sp.

different localities of Peshawar, 108 microbial strains were isolated. Among them, 15 were capable of hydrolysing cellulose; one belonged to *Pseudomonas* sp., one to *Aeromonas* sp., one to *Pasteurella* sp., and two belonged to *Staphylococcus* and ten to the genus *Bacillus* (Ahmad et al. 2013).

There are several reports on microbes capable of degrading cellulose in the mangrove environment. The microbes from the mangrove environment include bacteria, fungi, yeast and actinobacteria. Fifteen cellulose-degrading bacterial species such as *Micrococcus*, *Bacillus*, *Pseudomonas*, *Xanthomonas* and *Brucella* had been isolated from mangrove soils of Mahanadi River Delta (Odisha), India (Behera et al. 2014). Gao et al. (2012) reported isolation of a cellulolytic bacterium *Vibrio xiamenensis* from mangrove soil in Xiamen, Fujian province of China. Besides bacteria, fungi and actinobacteria were also capable of degrading cellulose in the mangrove environment. Luo et al. (2005) screened 29 fungal isolates from mangroves and other marine sources in Thailand, Hong Kong and Vietnam. Most of the fungal

isolates that produced endoglucanases were ascomycetes. Three fungal strains were isolated from Valanthakad mangroves, Kerala, India. Among the three, *Trichoderma viride* showed a high cellulase activity (Nathan et al. 2014).

Thermophilic microbes isolated from hot springs have been screened for cellulases. As most of the industrial enzymatic reactions are carried out at high temperature, cellulases isolated from such extreme conditions are of great value for industrial applications. Priya et al. (2016) isolated and screened eight bacterial strains from Tattapani hot spring, North Himalaya, India, for various hydrolytic enzymes including cellulases. The isolated bacterial strains were identified by 16S ribotyping as *Geobacillus* and *Bacillus* sp. The maximum cellulase activity was reported in *G. thermodenitrificans* among eight strains isolated. A novel enzyme CelDZ1 was produced by *Thermoanaerobacterium* isolated from hot spring in Grensdalur, Iceland. Biochemical characterization revealed that it is a GH5 cellulase with good thermostability and high halotolerance (Zarafeta et al. 2016). The microbial diversity of Y-shaped Sungai Klah hot spring located in Perak, Malaysia, was studied by Lee et al. (2018a). The microbial profiling data demonstrated that among the detected genera, at least 18 genera (*Acidimicrobium*, *Aeropyrum*, *Caldilinea*, *Caldisphaera*, *Chloracidobacterium*, *Chloroflexus*, *Desulfurobacterium*, *Fervidobacterium*, *Geobacillus*, *Meiothermus*, *Melioribacter*, *Methanothermococcus*, *Methanotorris*, *Roseiflexus*, *Thermoanaerobacter*, *Thermoanaerobacterium*, *Thermoanaerobaculum* and *Thermosipho*) were found in this natural ecosystem that are potential candidates for efficient lignocellulosic enzymes. A thermophilic cellulose-degrading consortium XM70 was developed from a hot spring in China. The presence of *Geobacillus*, *Thermus*, *Bacillus* and *Anoxybacillus* was observed along with genes encoding enzymes belonging to 15 glycoside families (Zhao et al. 2017).

Several investigations have been carried out on the isolation of microbes from gut microbiomes of animals and insects. Ruminants do not produce cellulolytic enzymes but rely on symbiotic microorganisms residing in their digestive tracts to produce enzymes for digesting lignocellulosic feed. Therefore, microorganisms present in the rumen of ruminants can be a promising source of cellulolytic enzymes (Krause et al. 2003). Song et al. (2017) isolated a novel GH74 cellulase from the black goat rumen metagenomic library. The protein showed 77% sequence identity with two endo-1,4 β-glucanases from *Fibrobacter succinogenes*. The protein was further overexpressed in *E. coli* and characterized. A fungal strain HDZK-BYTF620, which was identified as *Aureobasidium pullulans*, was isolated from the gut of termite species, *Odontotermes formosanu.* The activity of cellulase produced from the strain was characterized (Duan et al. 2017). A cellulolytic bacterium identified as *Bacillus subtilis* BY-2 based on morphological, physiological and biochemical characteristics as well as 16S rRNA sequence analysis was isolated from a Tibetan pig's intestine. Fermentation conditions were also found for maximum enzyme production. The initial pH of 5.5 with 4% inoculum resulted in a high titre of enzyme in 24–48 h with maximum CMCase activity of 3.56 U/mL (Yang et al. 2014).

Bacterial colonies were isolated from soil samples collected from Forest Park in South Korea. Only 21% of the isolates were found to be positive for cellulolytic activity. Strains with a high enzyme activity were selected and identified as *Bacillus*

subtilis, *B. amyloliquefaciens* and *B. atrophaeus* based on highest identity and 97% likelihood in the biochemical properties (Lee et al. 2016). The micro-fungal species *Fusarium oxysporum*, *Curvularia lunata*, *Alternaria alternata*, *Aspergillus niger*, *Cladosporium cladosporioides*, *Penicillium citrinum* and *Aspergillus nidulans* were isolated from soil and litter samples collected from Sathuragiri Hills (Western Ghats, Tamil Nadu, India). Among these, highest enzyme activity was recorded in *P. citrinum* (Muthukrishnan 2017).

It is widely known that bacteria and fungi are the reservoirs of cellulolytic organisms, but the role of protozoa and ciliates in biomass degradation has not been fully elucidated. There have been attempts to isolate and characterize ciliate genes using recombinant DNA technology. The most dominant clan reported to be present in the transcriptome of the flagellate community was GH7 (Todaka et al. 2007). The exoglucanase gene (PgCBH) from *Pseudotrichonympha grassii*, a flagellate found in the hindgut of wood-feeding termite *Coptotermes formosanus*, was isolated and characterized. The isolated gene showed similarity with GH7 family (Nakashima et al. 2002). The expression level of PgCBH was observed to be downregulated by RNAi leading to death of the host organism. The observations confirmed the importance of gene in lignocellulolytic process of protists and in host survival (Liu et al. 2017a). Wereszka et al. (2004) isolated and characterized a GH5 from the rumen ciliate *Epidinium caudatum.* The isolated gene was closely related to cellulase genes of rumen bacteria. The pH optimum was unusual at above 8 that suggests its role in the alkaline digestive vacuoles of its host. The cellulolytic activity of 15 species of entodiniomorphid protozoa was measured. The highest activity was found in *Eudiplodinium maggii*, *Epidinium ecaudatum caudatum* and *Ostracodinium obtusum bilobum*, while no activity was found in five *Entodinium* spp. (Coleman 1985). Similar observations were reported by Béra-Maillet et al. (2005). The endoglucanase activity of three rumen protozoa was assessed. Enzyme activity of *Polyplastron multivesiculatum* was two-fold higher than that in *E. maggii*, whereas weak activity was observed for *Entodinium* sp. (Béra-Maillet et al. 2005). A conclusive role of protozoa in the rumen and specifically fibre degradation is still not clear because it is not possible to maintain rumen protozoa in axenic culture. Metagenomic screening of protozoan glucosidases, however, has confirmed the presence of a diverse range of glycoside hydrolases in the rumen protozoa.

15.3 Diversity in Microbial Cellulases

Cellulolytic microbes produce considerable diversity of hydrolytic enzymes which are mainly expressed in cellulase-inducing conditions. Biomass degradation is achieved by the action of a complex mixture of enzymes comprising cellulases, hemicellulases, and ligninases, reflecting the complexity of the materials. Cellulases belong to glycoside hydrolase (GH) family. They are the key enzymes for the processing of complex carbohydrates. There are 61 GH families assigned to Pfam ID, according to CAZy database (http://www.cazy.org). More than 133 GH families are present in CAZy database, of which 16 families include cellulases. The current classification of glycosidase hydrolases is based on their amino

Table 15.2 Classification of various cellulases in different Glycoside Hydrolase (GH) families

Clan	GH family	Mechanism	3-D structure
GH-A	1, 2, 5, 10, 17, 26, 30, 35, 39, 42, 50, 51, 53, 59, 72, 79, 86, 113, 128, 147, 148	Retaining	$(\beta/\alpha)_8$
GH-B	7, 16	Retaining	β-jelly roll
GH-C	11, 12	Retaining	β-jelly roll
GH-D	27, 31, 36	Retaining	$(\beta/\alpha)_8$
GH-E	33, 34, 83, 93	Retaining	Sixfold β-propeller
GH-F	43, 62	Inverting	Fivefold β-propeller
GH-G	37, 63, 100, 125	Inverting	$(\alpha/\alpha)_6$
GH-H	13, 70, 77	Retaining	$(\beta/\alpha)_8$
GH-I	24, 80	Inverting	α + β
GH-J	32, 68	Retaining	Fivefold β-propeller
GH-K	18, 20, 85	Retaining	$(\beta/\alpha)_8$
GH-L	15, 65	Inverting	$(\alpha/\alpha)_6$
GH-M	8, 48	Inverting	$(\alpha/\alpha)_6$
GH-N	28, 49	Inverting	β-helix
GH-O	52. 116	Retaining	$(\alpha/\alpha)_6$
GH-P	127, 146	Retaining	$(\alpha/\alpha)_6$
GH-Q	94, 149	Inverting	$(\alpha/\alpha)_6$

acid similarity (Henrissat and Bairoch 1996).The cellulases belonging to GH families 5, 6, 7, 8, 12, 44, 45 and 48 act on cellulose. The remarkable diversity of cellulases appears to be due to the structural complexity and rigidity of cellulosic substrates. Table 15.2 presents the classification of various cellulases in different glycoside hydrolase clans and families with their different catalytic mechanism and 3-D structure. All members of a GH family have the same basic protein fold and utilize the same catalytic mechanism, but they differ only in their substrate specificities and amino acid sequences. Cellulose degradation is a complex mechanism, which requires the synergistic action of multiple GH family enzymes. The GH families 5, 6, 7, 8, 12, 44, 45 and 48 include three enzymes: (i) endoglucanases, which act on internal β-1,4-glycosidic bonds to produce cello-oligosaccharides; (ii) exoglucanases, which degrade cellulose matrix from extreme ends to yield cellobiose; and (iii) β-glucosidases, which generate glucose from cellobiose. The exoglucanases are also further classified: one attacks the non-reducing end and the other attacks the reduced end of cellulose matrix. All GH6 enzymes attack the non-reducing end. Enzymes from aerobic fungal members and bacterial members that attack reducing ends are placed in the family GH7 and GH48, respectively. Some anaerobic microbes possess cellobiose phosphorylase, also known as dextrin phosphorylase, which converts cellobiose and soluble dextrins to glucose and glucose-1-phosphate, conserving the energy in the cellobiose linkage.

The enzyme synthesis is primarily regulated at the transcription level. The mechanism of regulation of cellulase synthesis has been studied mainly in *Aspergillus* and *Trichoderma*. The synthesis of these enzymes is co-ordinately regulated and induced in the presence of specific substrate polymers. Various mono- and oligosaccharides such as sophorose (Mandels et al. 1962) and cellobiose (Mandels and Reese 1960) induce cellulase expression. The genes encoding hydrolytic enzymes are repressed in the presence of glucose (Hsieh et al. 2014). Various transcriptional factors such as XlnR, ManR, McmA and ClbR in *Aspergillus* and BglR, Xyr1 and Ace3 in *Trichoderma* have been reported to regulate the cellulolytic and/or hemicellulolytic enzyme production (Kunitake and Kobayashi 2017). Other factors such as light signalling and antisense RNA accumulation also play a critical role in enzyme regulation (Tani et al. 2014). The difference and cross talks between the expression of cellulase and hemicellulase genes have made regulatory mechanisms intricate. The complex and diverse regulatory mechanisms of cellulase synthesis would possibly reflect diversity in cellulases.

Berlemont and Martiny (2013) studied phylogenetic distribution of cellulases in bacteria. Members of several phyla, including *Firmicutes*, *Bacteroidetes*/*Chlorobi* group, *Fibrobacteres*, *Acidobacteria* and *Thermotogae*, have been reported to have more than five putative enzymes per genome. The enzymes from the families GH1 and GH3 are the most abundant, whereas GH6, 8, 9, 12, 44, 45 and 48 are less abundant across all bacterial genomes. GH6 was exclusively identified in *Actinobacteria* and *Proteobacteria.* Similarly genes associated with GH9 have been reported in some *Actinobacteria*, *Fibrobacteres*, *Firmicutes*, *Proteobacteria* and *Spirochaetes*. The genes associated with GH12 were found exclusively in *Actinobacteria*, *Thermotogae*, *Firmicutes* and *Proteobacteria*. The genes associated with GH44, 45 and 48 occur in *Actinobacteria*, *Firmicutes* and *Proteobacteria*. A study of the CAZy data reveals that GH1 and GH5 include cellulases of bacterial, fungal as well as archaeal origin. Fungal hydrolases are in families 30 and 48, while families 8 and 44 include only bacterial hydrolases.

Phylogenetic trees of various microbial endoglucanases (Fig. 15.1), cellobiohydrolase/exoglucanases (Fig. 15.2) and β-glucosidases (Fig. 15.3) belonging to different GH families from archaeal, bacterial and eukaryotic domains depict >80% sequence similarity. The branch length also depicts the proximity among cellulases.

The microbes evolved two strategies for the degradation of cellulose: (1) discrete non-complexed cellulases that are usually secreted by aerobic bacteria and fungi and (2) complexed cellulases (cellulosomes) that are usually expressed on the surface of anaerobic bacteria and fungi. Non-complexed cellulases involved in the secretion of a set of enzymes work synergistically for the breakdown of cellulose (Wilson 2008). Most of these cellulases comprise a carbohydrate-binding module (CBM) joined by a flexible linker to one end of the catalytic domain (Shoseyov et al. 2006). There are a few microbes which secrete cellulases and peroxidases (Martínez et al. 2005). The peroxidase produces peroxide that partially oxidizes cellulose, making it much easier for the cellulases to degrade it. Cellulosomes on the other hand are protrusions (stable enzyme complexes) produced on the cell wall of

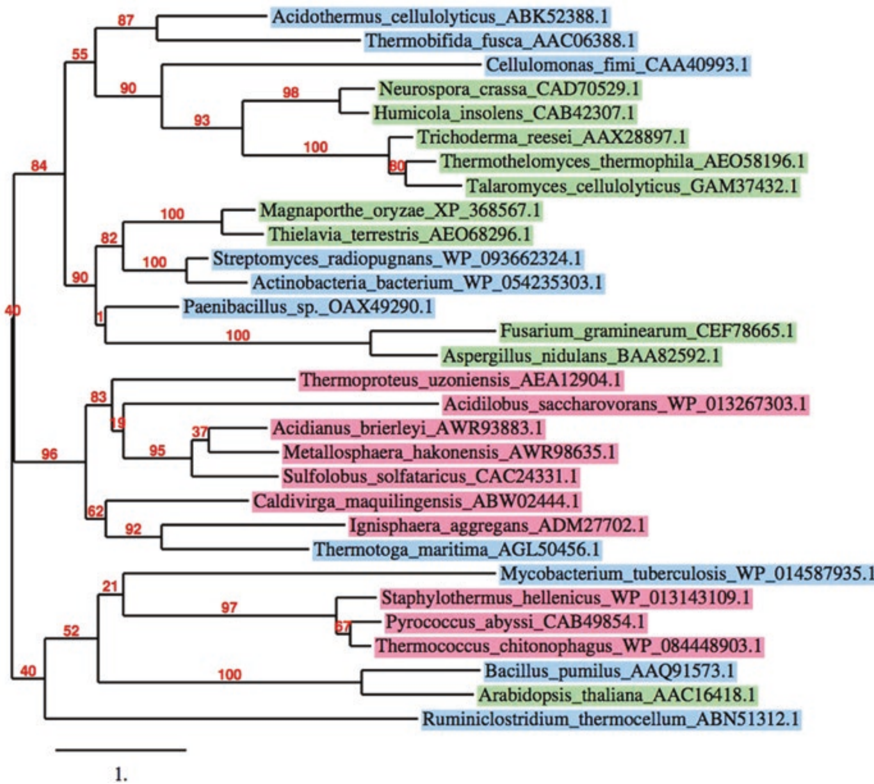

Fig. 15.1 Phylogenetic tree of endoglucanases: The bootstrap values (%) are mentioned besides the branch nodes. The names of organisms and accession numbers of the proteins are cited. The red highlighted species belong to archaea, blue highlighted species belong to bacteria, and green highlighted species belong to eukaryota domains

cellulolytic anaerobic microbes while growing on cellulosic materials. Besides these two mechanisms, there is another mechanism which is yet to be explored. A few microbes such as *Cytophaga hutchinsonii* and *Fibrobacter succinogenes* neither secrete free cellulases nor produce cellulosomes (Zhu et al. 2016; Suen et al. 2011), thus suggesting the presence of a novel cellulose-degrading mechanism, which is yet to be investigated.

The recent developments in metagenomics have facilitated identification of novel cellulases, which open up new avenues for their application in biorefineries. Various environmental niches have been explored for cellulase-encoding genes. The cellulolytic enzymes from rumen microorganisms principally fall within three families (GH5, 9 and 51). A GH5 cellulase gene (*Cel PRII*) from buffalo rumen metagenome was cloned in *E. coli*, expressed and characterized. The enzyme was stable at pH 10.0 and 60 °C (Shah et al. 2017). A novel thermostable cellulase had also been isolated from oil reservoir metagenome by Lewin et al. (2017). The metagenomic fosmid library was constructed, and the clones were screened for the expression of

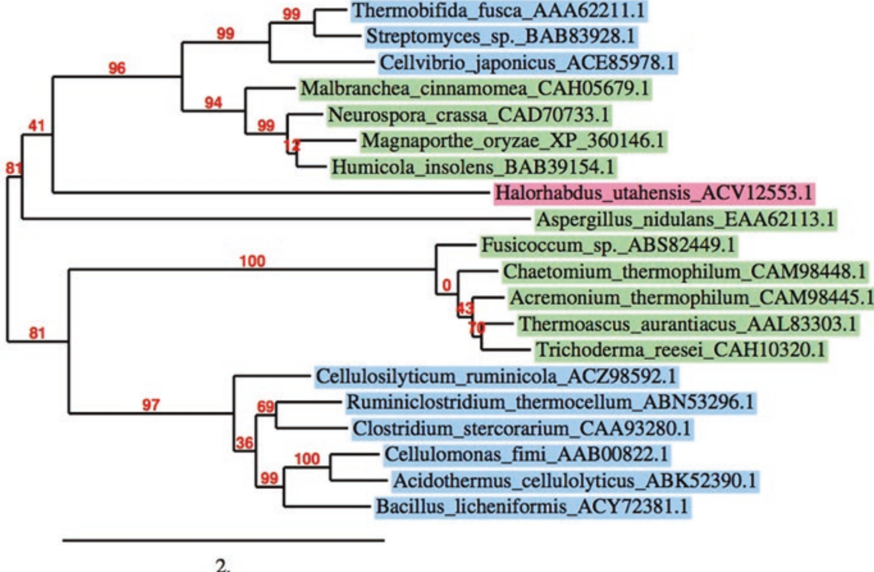

Fig. 15.2 Phylogenetic tree of exoglucanases: The bootstrap values (%) are mentioned besides the branch nodes. The names of the organisms and accession numbers of the proteins are cited. The red highlighted species belong to archaea, blue highlighted species belong to bacteria, and green highlighted species belong to eukaryota domains

carbohydrate-degrading enzymes. Out of five, three of them were highly thermostable, suggesting thermophilic archaeal origin. The sludge metagenome was explored for retrieving genes that encode carbohydrate-active enzymes. Based on the hidden Markov model, 253 genes encoding thermostable putative carbohydrate-active enzymes were identified. The majority of the genes belonged to GH9 and the corresponding CBM3 (carbohydrate-binding module) [Xia et al. 2013]. Cellulases with activity against oil palm empty fruit bunch (a byproduct of the palm oil industry) were identified from a metagenomic library constructed from a high-Andean forest ecosystem. The three families of cellulases have been identified based on the sequence analysis of the inserts. Multiple alignment data depicted metagenomic sequences most probably derived from bacteria (Medina et al. 2017). Since the majority of microbes present in the environment are non-culturable, metagenomics enables retrieval of genes from environmental DNA encoding novel biocatalysts for a wide range of applications.

Recent research efforts have indicated the presence of accessory enzymes/proteins such as swollenin, expansin-like proteins and lytic polysaccharide monooxygenases (LPMOs) which boost the hydrolytic performance of cellulases (Hu et al. 2013; Eibinger et al. 2014). LPMOs are copper-containing oxygenases, which oxidize recalcitrant polysaccharides such as cellulose, thereby allowing substrate accessibility for the enzyme action (Walton and Davies 2016). LPMOs boost the hydrolytic breakdown of lignocellulosic biomass, particularly cellulose, due to their

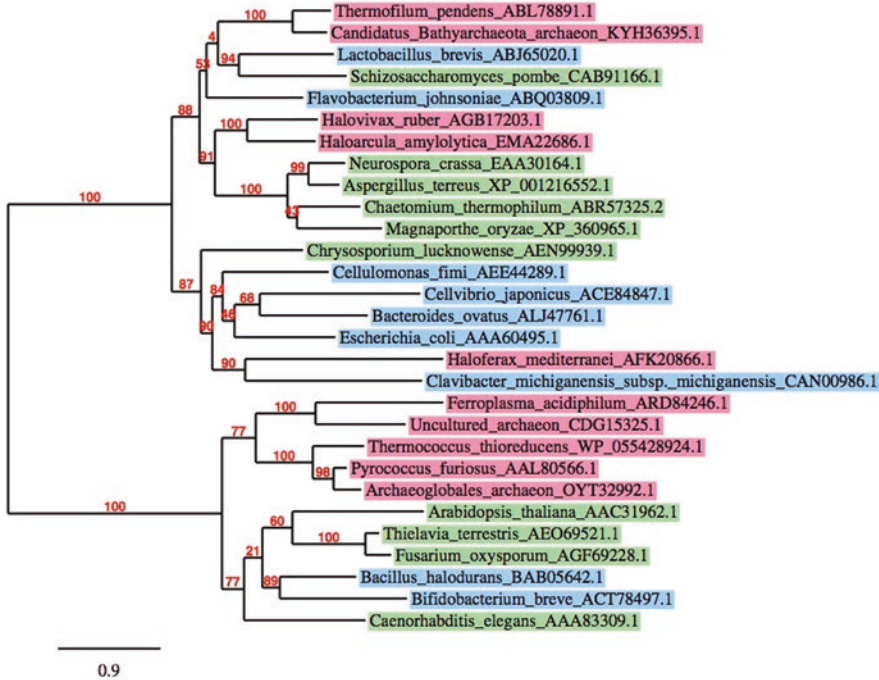

Fig. 15.3 Phylogenetic tree of β-glucosidases: The bootstrap values (%) are mentioned besides the branch nodes. The names of organisms and accession numbers of the proteins are cited. The red highlighted species belong to archaea, blue highlighted species belong to bacteria, and green highlighted species belong to eukaryota domains

oxidative mechanism. LPMOs are reclassified in the CAZy database from GH61 and CBM33 to AA9 and AA10, respectively (Levasseur et al. 2013). Cellulose conversion was enhanced from 46% to 54% by supplementing CBH I (*Tl*Cel7A) from *T. longibrachiatum* with LPMO (*Tr*AA9A) of *T. reesei* (Song et al. 2018). When LPMO (*Gt*GH61) from *Gloeophyllum trabeum* was added to xylanase (*Gt*Xyl10G) or cellulase (*Gt*Cel5B), the hydrolysis rate increased by 56 and 174% in the pretreated kenaf and oak, respectively (Jung et al. 2015). The addition of LPMOs to cellulolytic and hemicellulolytic enzymes, therefore, appears to be a novel approach for improving the rates of saccharification of cellulosics (Müller et al. 2015).

15.4 Characteristics of Microbial Cellulases

The characterization of cellulases has been reported in terms of molecular mass, optimal pH and temperature, thermostability and glycosylation. Usually, the cellulases are known to be monomeric in nature, although some β-glucosidases are dimeric. The molecular mass of cellulases ranges between 30 and 250 kDa. The cellulases from thermophilic fungi are active in the pH range 4.0–7.0 and

temperature between 50–80 °C. They are known to have remarkable thermostability at higher temperatures with longer half-lives. Therefore, cellulases have been exploited in various industrial processes due to their high thermostability, as this is one of the crucial factors required in industrial processes.

Most of the fungal cellulolytic enzymes are usually acidic in nature with pI values between 2.0 and 6.0 and a maximal catalytic activity at pH 4.0 to 6.0. The optimal temperatures for purified endoglucanase (EG) during carboxymethyl cellulose (CMC) hydrolysis are approximately 50 and 60 °C. The substrates used for EGs are CMC, amorphous cellulose, crystalline cellulose and cello-oligomers (cellotetraose and cellotriose). The cellobiose and palladium complexes are known to strongly inhibit EGs.

The maximum enzyme activity of purified cellulase from *Geobacillus* sp. was recorded at 60 °C, and more than 50% of enzyme activity was observed at pH 3.0 to 9.0 (Potprommanee et al. 2017). Endoglucanase produced from *Aspergillus terreus* on rice straw in solid-state fermentation was of 28.18 kDa and the maximum activity at 50 °C and pH 4.8. The enzyme was thermostable at 60 °C for 120 min (Narra et al. 2014). Elshafei et al. (2009) reported that *A. terreus* DSM826 EG is stable at 50 °C for 1 h without apparent loss of activity. The EG from *Bacillus pumilus* EB3 found to retain 66% of its optimal activity at 70 °C (Ariffin et al. 2008). The low molecular mass of purified EGs from other *Aspergillus* spp. has been reported (Akiba et al. 1995; Chen et al. 2007). The optimum temperature and pH of cellulase isolated from *Bacillus vallismortis* RG-07 were 65 °C and 7.0, respectively, although the enzyme retained 95 and 75% activity at 95 °C and pH 9.0. The enzyme activity was enhanced in the presence of a few organic solvents, Ca^{2+}, β-mercaptoethanol, Tween-60 and sodium hypochlorite, but strongly inhibited by mercury (Gaur and Tiwari 2015). The endoglucanase gene from *Myceliophthora thermophila* (Phadtare et al. 2017) and *Chaetomium thermophilum* (Hua et al. 2018) had been heterologously expressed in *Pichia pastoris*. It was monomeric with molecular mass of 47 kDa. The optimum activity was recorded at pH 10 and 50 °C for EG from *M. thermophila*, whereas it was at pH 5.0 and 55 °C for EG from *C. thermophilum*. The $t_{1/2}$ of EG from *M. thermophila* was 60 and 15 min at 90 and 100 °C, respectively. The homology modelling showed catalytically important role of glutamate 234 and 344 (Phadtare et al. 2017). Table 15.3 presents the characteristics of recently characterized EGs from various sources.

Volkov et al. (2014) reported the homologous cloning of *CBH* (Cel7A) from *Penicillium canescens*. The molecular mass of 80–90 kDa with pI 4.5 was recorded for the recombinant CBH. Glycosylation of CBH is none or low <12 %, like endoglucanases (Eriksson and Pettersson 1975; Schmidhalter and Canevascini 1993; Hamada et al. 1999). The CBHs are usually monomeric with a molecular mass typically ranging between 36 and 65 kDa. The pI values of CBHs are acidic, typically between 3.6 and 5.0. The recombinant enzyme (Cel7A) exhibited maximum activity at pH 4.0–4.5 and was stable at 50 °C for 3 h, while at 60 °C, it lost 45% activity after 30 min of incubation. Using peptide mass fingerprinting, N-terminal Gln26 residue was found in the form of pyroglutamate, which is known to improve the enzyme activity and stability. The characteristics of a few recently characterized CBHs are presented in Table 15.4.

Table 15.3 Characteristics of endoglucanases (EGs)

Gene/enzyme	Organism	Molecular mass (kDa)	Optimal temp. (°C)	Optimal pH	pI	K_m	V_{max}	References
Endoglucanase	*Photobacterium panuliri*	53	40	4	NR[a]	0.76%	29.32	Deep et al. (2016)
pcGH5	*Phanerochaete chrysosporium*	43	50	4	NR	3.7	20.8	Huy et al. (2016)
Endoglucanase	*Ganoderma lucidum*	64	35	5	NR	NR	NR	Manavalan et al. (2015)
Sprcel8A	*Serratia proteamaculans*	41.2	40	7	5.37	6.87	3.5	Cano-Ramírez et al. (2016)
AfmE1 (GH8 EGL)	*Raoultella ornithinolytica*	39	45	6–6.5	NR	8.6	74.9	Scapin et al. (2017)
PHS	Hot spring sediment metagenome	60	65	8	NR	3.87	370.37	Gupta et al. (2017)
AtGH12	*Aspergillus terreus*	24	55	5	4.25	2.214	562	Segato et al. (2017)
KG37 (GH5 EG)	Goat rumen metagenome	35	30–50	6–7	NR	NR	NR	Song et al. (2017)
mgCel6A	Compost metagenome	45	85	6	NR	NR	NR	Jensen et al. (2018)
FpCel45	*Fomitopsis palustris*	30	80	5	NR	NR	NR	Cha et al. (2018)

[a]*NR* not reported

Table 15.4 Characteristics of cellobiohydrolases (CBHs)

Gene/ enzyme	Organism	Molecular mass (kDa)	Optimal temp. (°C)	Optimal pH	pI	K_m	V_{max}	References
CBH 1.2	*Humicola grisea*	47	60	8	NR[a]	NR	NR	Oliveira et al. (2013)
Cel A CBH	*Neocallimastix patriciarum* J11	55	50	6	NR	NR	NR	Wang et al. (2013)
Cel 7A CBH	*Penicillium canescens*	90	50	4.5	4.5	1	0.7	Volkov et al. (2014)
Cel7A CBH	*Geotrichum candidum*	75	50	5	NR	1	NR	Borisova et al. (2015)
CBH7 B	*Thielavia terrestris*	51.8	55	5	NR	402	0.28	Woon et al. (2015)
GH48 CBH	*Streptomyces coelicolor* A3	103.4	50	5	NR	NR	NR	Lee et al. (2018b)
Cel6H	Compost metagenome	35	50	5.5	NR	1.3	8.75	Lee et al. (2018c)
Cel6H	Compost metagenome	23	50	5.5	NR	1.32	6.17	Lee et al. (2018c)

[a]*NR* not reported

β-Glucosidases (BGLs) are monomeric, dimeric or even trimeric proteins. They act on cellobiose and release glucose molecules. The activities of BGL are typically measured using substrates such as 4-nitrophenyl β-D-glucopyranoside (pNPG), cellobiose and sophorose. The BGLs can be extracellular, cell wall-associated and intracellular. The molecular masses of BGLs typically vary between 35 and 640 kDa (Baldrian and Valášková 2008). In some studies, BGLs are reported to be glycosylated up to 90% of molecular weights (Dashtban et al. 2010). The extracellular BGLs have been reported to be acidic with pI of 3.5 to 5.2. The intracellular BGLs generally are neutral with pI of 6.2 to 7.0. The optimum pH values of BGLs are usually in the range of 3.5 to 5.5. However, there are some reports which state neutral pH optimum for some BGLs. Like other cellulases, the optimal temperatures for BGLs are between 45 and 75 °C. Cello-oligosaccharides are generally used as substrates for BGLs, but the enzymes are inactive on crystalline cellulose and display only low activity on amorphous high molecular mass cellulose (Sadana et al. 1983; Valášková and Baldrian 2006).

The gene encoding BGL from *Bacillus licheniformis* was cloned and expressed in *E. coli* BL21 by Chen et al. (2017). The recombinant BGL (Bgl.bli1) was of 53.4 kDa by SDS-PAGE. The activity of the recombinant Bgl.bli1 was optimal at pH 7.0 and 60 °C. The observations suggested that the enzyme is thermotolerant, as it retained approximately maximum activity at 80 °C. The enzyme activity was markedly increased by the presence of Zn^{2+}, Mg^{2+} and Mn^{2+} ions, while Cu^{2+} and Ca^{2+}

significantly inactivated the BGL (Chen et al. 2017). The recombinant BGL was found to be inhibited by glucose, as reported by other groups (Smith and Gold 1979). A BGL cloned in *E. coli* from *Jeotgalibacillus malaysiensis* (mesophilic and halophilic) was 52 kDa. The enzyme was optimally active at 65 °C and retained ~95% of the maximal activity at 70 °C. The activity and stability of recombinant BGL was enhanced by calcium chloride (Liew et al. 2018). Table 15.5 presents characteristic features of some recently characterized BGLs.

A very few attempts have been made to characterize the LPMOs. Kojima et al. (2016) expressed a LPMO in *P. pastoris* from a brown rot fungus *Gloeophyllum trabeum* and characterized it. The molecular mass of recombinant protein (GtLPMO9A-2) was 60 kDa. The higher MW than estimated amino acid sequence suggests that the recombinant enzyme was *N*- and *O*-glycosylated. GtLPMO9A-2 was observed to act preferentially on free xyloglucan and have a broad substrate specificity compared to other characterized LPMOs. A LPMO from *Myceliophthora thermophila* was cloned in *P. pastoris*, and its synergism with EG was reported by Karnaouri et al. (2017). The addition of LPMO to EG enhanced enzymatic hydrolysis. A chitin-active LPMO (JdLPMO10A) from *Jonesia denitrificans* was structurally and functionally characterized. The considerably small molecular mass of protein (15.5 kDa) was reported. The overall structure analysis showed a β-sandwich fold consisting of two β-sheets formed by seven β-strands (Mekasha et al. 2016). The functional characterization of E7 LPMO from *Thermobifida fusca* was done. LPMO from *Bacillus atrophaeus* (BatLPMO10) was cloned in *B. subtilis*. The pH optimum was at pH 8.0. The enzyme was inhibited by sodium ions (Yu et al. 2016). To understand the biochemical properties and to develop highly active LPMOs, it is essential to develop a quantitative assay system for them. Although there are a few assays available, but it is difficult to assay LPMO alone. Therefore, the characterization of LPMO remains a challenge.

15.5 Role of Cellulose-Degrading Microbes in Ecosystem Sustainability

Cellulose is the most abundant polysaccharide of plant cell walls, comprising almost 50% of the biomass synthesized by photosynthetic fixation of carbon dioxide (Eriksson et al. 1990). Cellulases in soils are derived mainly from bacteria and fungi present in plant debris. Mangrove communities provide large quantities of organic matter; therefore, they are rich in energy. They also possess a large active microbial population (Behera et al. 2017). The enzymes present in soil play a key role in maintaining soil health and fertility. They also have significant effects on soil biology, environmental management, plant growth and nutrient uptake. The microbes present in soil depend upon the carbon source for their growth and survival (Deng and Tabatabai 1994). Cellulases degrade the cellulose present in cell walls of plant debris into glucose that provides energy for microbes (Esen 1993; Tabatabai 1994). The cellulases are sensitive to changes in pH and can be used as a good biochemical indicator. Therefore, ecological changes resulting from soil acidification can also be measured using these enzymes.

Table 15.5 Characteristics of β-glucosidases (BGLs)

Gene/enzyme	Organism	Molecular mass (kDa)	Optimal temp. (°C)	Optimal pH	pI	K_m	V_{max}	References
UeBgl3A	*Ustilago esculenta*	110	40	5	NR[a]	7.2	NR	Nakajima et al. (2012)
PaBGL (GH3 BGL)	*Pholiota adiposa*	60	65	4	4.7	2.23	4390	Jagtap et al. (2013)
Cel3E BGL	*Penicillium piceum*	80	60	5	NR	0.0019	0.8	Gao et al. (2014)
bgl	*Thermoanaerobacterium aotearoense*	46	60	6	NR	0.66	180.6	Yang et al. (2015)
GH3BGL (mtbgl3b)	*Myceliophthora thermophila*	130	60	5	NR	2.78	927.9	Zhao et al. (2015)
DT-Bgl	*Anoxybacillus* sp. DT 3-1	53	70	8.5	NR	0.22	923.7	Chan et al. (2016)
Bgl.blil BGL	*Bacillus licheniformis*	53.4	60	4/5	NR	NR	NR	Chen et al. (2017)
BGL50	*Myceliophthora thermophila*	50	50/60	4.5/5	NR	0.42	0.52	Bonfá et al. (2018)
GH3 BGL	*Bifidobacterium adolescentis*	80	45	6.5	NR	0.32	0.37	Florindo et al. (2018)
BGLA1/A2/A3	*Aspergillus* YDJ 14	A1 – 100	A1 – 60	A1– 4	NR	A1 – 0.25	A1 – 131	Oh et al. (2018)
		A2 – 45	A2– 60	A1 – 4	NR	A2 – 0.27	A2 – 101	
		A3 – 40	A3 – 50	A3 – 5	NR	A3 – 1.26	A3 – 213.5	

[a]*NR* not reported

Cellulases are also one of the most important exogenous enzymes that are found in symbiotic relation between invertebrates and microbes. The invertebrates lack the ability to produce endogenous cellulases; thus, they rely on gut microflora to degrade the cell wall of vascular plants. The cellulase activity has been observed in alimentary tract of fishes too, which allow them to easily ingest algae, sea grass and other macrophytic plants. Besides cellulose degradation, cellulases from gut microbes increase the total amount of amino nitrogen. In some species, the gut microflora is known to carry out nitrogen fixation, thus increasing the nitrate concentration (Fong and Mann 1980). The symbiotic associations of cellulolytic eukaryotic protists and diverse bacteria have been reported in the gut microbial communities of termites (Ohkuma et al. 2015). Acetogenesis and nitrogen fixation are the two mechanisms performed by gut microbes in the host termite.

Numerous genes have been identified in human gut microbiome, but genes coding CAZymes are of specific interest as these enzymes are required to digest the complex dietary polysaccharides. A mini microbiome with reference genome was developed to explore the CAZyme profiles of the human gut microbiome (Kaoutari et al. 2013). It was observed that a total of 15,882 different CAZyme genes were distributed unevenly between GH and other families. Of which, 9120 genes encoding GHs have been found, which represent the majority (59%) of CAZyme genes (Kaoutari et al. 2013). Human genome studies revealed that it encodes 97 GHs, with only 17 enzymes known to breakdown carbohydrate nutrients (Cantarel et al. 2012).

Cellulases from few fungi are capable of degrading the cell walls of plant pathogens; hence, they play a role in the control of plant diseases. Therefore, fungal strains and their enzymes act as biocontrol agents to protect the plants from pathogenic attack. Recombinant *Trichoderma longibrachiatum* (Migheli et al. 1998) and *T. harzianum* (Thrane et al. 2000) have been reported to display biocontrol activity against *Pythium ultimum*. Some cellulolytic fungi such as *Trichoderma* spp., *Gliocladium* spp., *Chaetomium* spp. and *Penicillium* spp. are known to play a vital role in enhanced seed germination, rapid plant growth and flowering, improved root system as well as increased crop yields (Bailey and Lumsden 1998; Harman and Kubicek 1998). Recently, it has been reported that cellulases play a role in endosperm cap weakening and radicle elongation during lettuce seed germination (Chen et al. 2014).

Cellulases are known to induce cellulose synthesis in plants. A membrane-associated Korrigan (*Kor*) gene of *Arabidopsis thaliana* encodes a member of the endo-1,4-β-D-glucanase family. *Kor* acts in the cellulose synthase complex (CSC) as a regulatory component. Not much is, however, known about interaction between the CSC and *Kor* (Szyjanowicz et al. 2004; Desprez et al. 2007). Biochemical, cellular and developmental functions of various cellulase genes may provide insights into the molecular mechanisms involved in the construction of cell wall and control of cell elongation in plants. It has also been suspected to be involved in modifying plant cell walls and cell elongation (Nicol et al. 1998; Sato et al. 2001). The mutation in *Kor* gene has been reported to cause aberrant cell plate formation, incomplete cell walls and multinucleated cells, which leads to the development of severely abnormal seedling morphology. It is also known to play a critical role in cytokinesis (Zuo et al. 2000).

Robledo et al. (2008) investigated the role of *Rhizobium* endoglucanase (*CelC2*) in symbiotic root hair infection of legumes. The enzyme has a role in degrading the cell wall of root tip, which allows penetration of microsymbiont. *CelC2* is known to mediate the length of cellulose fibrils, which leads to biofilm formation (Robledo et al. 2012). Therefore, cellulases have both colonization and infection functions in *Rhizobium*.

β-Glucosidase is also a commonly found enzyme in soils and is widely distributed among plants, animals, fungi, bacteria and yeasts (Veena et al. 2011). It is also involved in decomposition of cellulosic plant debris to glucose. A novel β-glucosidase (LamN) from *Vibrio campbellii* was found to have a role in carbon cycling and the exploitation of marine detritus produced in phytoplankton blooms (Wang et al. 2015). β-Glucosidases are useful as a soil quality bio-indicator, which may provide information about the past biological activity and the capacity of soil to stabilize the soil organic matter (Bandick and Dick 1999; Ndiaye et al. 2000). The β-glucosidase is also known to be inhibited by heavy metal contamination (Joachim and Patrick 2008). Thus, the enzyme contributes to soil quality testing (Bandick and Dick 1999). The physical, chemical and biological properties of the soil can also be assessed by the presence of these enzymes in soil (Turner et al. 2002), easing the agricultural soil management strategies (Joachim and Patrick 2008). Therefore, better understanding of cellulases may contribute significantly to the studies of ecosystem sustainability.

15.6 Potential Applications of Cellulolytic Microbes and Their Cellulases

Cellulases are one of the widely used enzymes in the industry since their introduction (Xia and Cen 1999). Initially, it was investigated for the bioconversion of biomass to ethanol. It has now enormous application in waste management, bioethanol industry, animal feed industry, agricultural industry, laundry and detergent industry, textile industry, paper and pulp industry, wine and brewery industry, food processing industry, olive oil extraction, carotenoid extraction, pharmaceutical and medical sciences, protoplast production, genetic engineering and pollution treatment.

15.6.1 Bioethanol Industry

The most common application of cellulase is in the conversion of lignocellulosic biomass (corn cob, sugarcane bagasse, paddy straw and other agricultural residues) to bioethanol. Bioconversion of lignocellulosic biomass into useful and higher-value products typically requires multistep processes. These processes include pre-treatment of biomass (mechanical, chemical or biological), hydrolysis of the cellulosic biomass to monomeric sugars (hexose or pentose sugars) in the presence of cellulases, fermentation of these monomeric sugars in the presence of ethanologenic yeast strains and finally separation and purification of the desired products.

There are various factors that limit cellulase efficiency during hydrolysis process including biomass structure recalcitrance, end product inhibition, thermal deactivation of the native protein, nonspecific binding to lignin and irreversible adsorption of the enzymes to the heterogeneous substrate. Therefore, some of the technologies need to be improved. Investigations are underway to reduce the enzyme cost in the production of bioethanol. The optimization of cellulase production, immobilization of cellulases, protein engineering and directed evolution are a few approaches that aid in the development of efficient cellulases. Several pilot plants have been constructed till date in various countries such as Australia, Brazil, Canada, China, Denmark, Germany, Italy, India, Japan, Russia, Spain and the USA. Companies such as DuPont, Diversa, Novozymes and Dyadic are manufacturing enzymes in order to produce cellulosic ethanol in the future.

15.6.2 Animal Feed Industry

Cellulases are also known to improve feed value and performance of animals; therefore, its applications in feed industry have received considerable attention. They are known to hydrolyse the anti-nutritional components present in feed grains into easily absorbable ingredients, thus improving animal health and performance (Nawaz et al. 2016). Peiji and group studied 18 microbial strains and identified that *Chaetomium cellulolyticum* degrades cellulose and hemicellulose efficiently among other isolated strains. Solid layer fermentation of *C. cellulolyticum* on raw corn straw resulted in threefold enhancement in amino acid content, thus increasing feed value (Peiji et al. 1997). The application of thermostable cellulases in feedstock at elevated temperatures results in inactivating pathogens by degrading their cell wall (Benitez et al. 1998). Cellulase application also enhances digestibility and nutritional feed value, thereby eliminating the need of two-step processes. Greater milk yield, energy content and density were observed in French Alpine goats, fed with cellulase-enriched diet as compared to those fed without cellulase (Rojo et al. 2015).

15.6.3 Textile Industry

Enzymatic treatment has emerged as an environment-friendly solution to textile industries. Traditionally, the biostoning of jeans was done using chemicals such as sodium hypochlorite or potassium permanganate also known as pumice stones. The pumice stones have been replaced by cellulase-based treatment. The advantages include less damage of fibres, increased productivity of the machines and less work intensive (Araújo et al. 2009). The enzymes are helpful in loosening the dye, which can be easily removed by mechanical abrasion in the wash cycle. Cellulases (endoglucanases) are also used in biopolishing and biofinishing of fabrics to enhance fabric look, fuzziness, feel and colour, thus eliminating the need of any chemical coating of fibres (Saravanan et al. 2013).

15.6.4 Paper and Pulp Industry

Mechanical paper pulping involves high energy consumption; therefore, the use of cellulases provides less energy-intensive as well as eco-friendly approach. Cellulases have been used for the biomodification of fibre properties. Cellulase treatment decreased the pulp viscosity and defibrillation, thereby reducing the fibre coarseness; it is also known to enhance the bleachability of kraft pulp. Cellulases are useful in deinking of different types of paper wastes, in combination with xylanases or alone (Chutani and Sharma 2016). The main advantage of enzymatic deinking process is lower usage of alkali and other deinking chemicals, improved fibre brightness (Vyas and Lachke 2003), enhanced strength properties, high pulp freeness and cleanliness. The cellulase treatment is also known to reduce water vapour retention rate and in producing grease-resistant paper (Lu et al. 2016). Thus, adverse environmental effects of paper industries could be lowered using enzymatic processes. Cellulases have potential use in the production of biodegradable cardboard (Salonen 1990) and manufacturing of soft paper (Gu and Huynh-Ba 2017) and tissues (Hsu and Lakhani 2002).

15.6.5 Wine and Brewery Industry

Cellulases have the most common application in fermentation processes to produce alcoholic beverages including beers and wines. The glucanases are added during fermentation to hydrolyse glucan and reduce the viscosity of wort that leads to improvement in the quality and yields of fermented products. The enzymes also play an important role in improving colour extraction, skin maceration, must clarification and filtration. β-Glucosidases are also known to improve the aroma of wines by modifying glycosylated precursors (Gil and Vallès 2001).

15.6.6 Food Processing Industry

Cellulases are widely used in food industries in numerous processes like fruit and vegetable juice clarification along with other enzymes as a part of macerating enzyme complex to increase juice yield, reducing the viscosity of nectars, concentrating purees, alteration of fruit sensory properties and improving the quality of bakery products (Sharma et al. 2014). The bitterness of citrus fruits and vegetables, their texture, flavour and aroma properties can be improved by infusion of β-glucosidases. Enzyme mixtures containing pectinases, cellulases and hemicellulases are used for improved extraction of colour and flavour from spices and other plant materials and oil from olive, sunflower and rapeseed (Sowbhagya and Chitra 2010).

Cellulases have a role in baking industry as well. Liu et al. (2017b) reported that cellulase supplementation increased the development time, stability, extensibility and stickiness of regular dough and decreased both softening and resistance to

extension. The combination of other enzymes such as α-amylase and xylanase with cellulase was observed to have a synergetic effect on the dough rheology.

15.6.7 Pharmaceutical Industry

The application of cellulases in prebiotics is emerging as a novel prospect. Prebiotics in diet include non-digestible fibres, which pass upper gastrointestinal tract and act as the substrate for growth of useful bacteria in the large intestine. The depolymerization of glucomannans can be attained by the action of cellulases and mannanases, and glucomannan hydrolysates thus produced are reported to promote growth of lactic acid bacteria (Al-Ghazzewi and Tester 2012). Cellulases are, therefore, valuable as prebiotics which can be added to a wide range of foods, feeds and healthcare/pharmaceutical products (Al-Ghazzewi et al. 2007). Cellulase has been used in processing of fruits, vegetables and cereals. A pharmaceutical company in India (Yakult Pharmaceutical Industry Co. Ltd.) uses cellulase from *T. viride* and *A. niger* for this purpose (http://www.yakult.co.jp/ypi/en/product.html). Cellulase has been used in releasing medicinally relevant compounds and extraction of bioactive polyphenols from plants or plant products. Enzymatic digestion using cellulases was efficient in extracting polyphenols from grape pomace, which can be utilized as source of natural antioxidants (Kabir et al. 2015).

15.6.8 Biorefinery

The production of biochemicals, energy and value-added products from lignocellulosic biomass is known as biorefinery concept. This is the most striking application of cellulases in the utilization of lignocellulosic waste in an economically profitable manner. Companies such as Iogen, POET and Abengoa are building refineries that can utilize biomass and convert it into ethanol. The production of chemicals such as lactic acid, succinic acid and citric acid using cellulases from food residues has been described extensively (Kiran et al. 2014). A unified approach utilizing the combination of cellulases and amylases involving anaerobic digestion and aerobic fungal fermentation has been developed for the conversion of corn stover and animal manure to biodiesel. The integrated process generated 1 L biodiesel and 1.9 kg methane from 12.8 kg dry dairy manure, 3.1 kg dry food wastes and 12.2 kg dry corn (Zhong et al. 2015). Available lignocellulosic biomass can be used in biorefinery concept. A major emphasis has been laid on the optimization of various processes involved, higher recovery and yield of value-added products.

15.7 Conclusions and Future Perspectives

Cellulose-degrading prokaryotic as well as eukaryotic microbes occur in both terrestrial and aquatic environments. They produce main (exoglucanase, endoglucanase and β-glucosidase) and accessory (swollenin, LPMO and others) cellulolytic

enzymes for degrading cellulose. Cellulases play a role not only in cellulose degradation but also in cellulose biosynthesis. There is a need to understand mechanisms of regulation of cellulase synthesis. Cellulases are also known to have a role in various ecosystem processes. Although cellulase-encoding genes have been cloned and expressed from various microbes in both prokaryotic and eukaryotic hosts, it is not often possible to attain high production levels. Further investigations are called for understanding the reasons for not being able to attain high production levels. The use of enzyme cocktails in hydrolysis of biomass is an emerging aspect in the field of lignocellulosic bioethanol. The blend of various cellulolytic and hemicellulolytic enzymes along with other accessory enzymes can prove beneficial in achieving maximum cellulose hydrolysis and fermentable sugar yields. Although impressive developments have been made in bioethanol production, further efforts are called for bringing down the cost of the enzymes in order to achieve substantial reduction in the cost of second-generation bioethanol.

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16 New Paradigm in Degradation of Lignocellulosic Biomass and Discovery of Novel Microbial Strains

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Abstract

Microbial degradation of lignocellulosic biomass (LCB) is of great human interest as it results in the production of numerous value-added products. 2G ethanol is an important lignocellulosics-based product that offers a long-term biotechnological solution to the depleting crude oil reserves without competing with food resources. The efficient degradation of LCB requires synergistic action of an array of microbial enzymes that include modular and non-modular glycosyl hydrolases (endoglucanase, exoglucanase, β-glucosidase, endoxylanase, β-xylosidase, α-arabinofuranosidase, α-glucuronidase, α-galactosidases and β-mannosidases), carbohydrate esterases (CE proteins) and other auxiliary enzymes (LPMOs, CDH and laccases). The genomics and proteomics studies have suggested a variety of culturable and non-culturable lignocellulolytic microorganisms inhabiting diverse ecological niches such as decaying plant materials, soil, compost piles, rumens, forest waste piles, wood processing plants, methanogenic sludge and surface of seashore. Among the rich microbial diversity, fungi are known for their ability to produce copious amounts of these lignocellulolytic enzymes. The wild-type fungal strains (with low specific activities) are subjected to several strain improvement strategies employing cyclic mutagenesis, recombinant technologies and other molecular techniques targeting regulatory elements to enhance their enzyme titres, specific activities and catalytic/hydrolytic efficiencies. The cellulase-/hemicellulase-rich preparations produced by growing developed strains on inexpensive agro-residues as carbon sources (under SSF and/or SmF) find potential applications in biorefineries, paper and pulp industry, animal feed, food and beverages industry and textile and detergent industry, making global business of USD 800 million per year.

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16.1 Introduction

Lignocellulosic biomass (LCB) has started showing enormous potential to meet escalating energy demand worldwide, especially in the form of liquid transportation fuel. LCB is the most abundant and inexpensive source of renewable energy on the earth (Gusakov 2011; Prasad et al. 2018) that could be processed into biofuel without competing with food production. LCB constitutes inedible proportions of the agricultural waste (rice straw, wheat straw, sugarcane bagasse, corn cob, corn stover, barley husks, etc.), grasses, waste paper, food industry residue and municipal solid wastes (Roig et al. 2006; Sánchez 2009). The straws and grasses have a pliable structure with low lignin content which makes them relatively easy to hydrolyse as compared to woody biomass (Wijaya et al. 2014). The major components of the agro-residue-based LCB are cellulose (35–40%), hemicellulose (25–35%) and lignin (12–18%) which account for 80% of the total weight of raw materials (Sánchez 2009) (Fig. 16.1). Besides these three major components, low molecular weight carbohydrates, pectins, proteins, lipids, water, ash, etc. constitute minor components. The distribution of cellulose, hemicellulose and lignin varies significantly among different plants and between different plant parts as well. Furthermore, the complexity of structure in terms of spatial distribution also varies significantly.

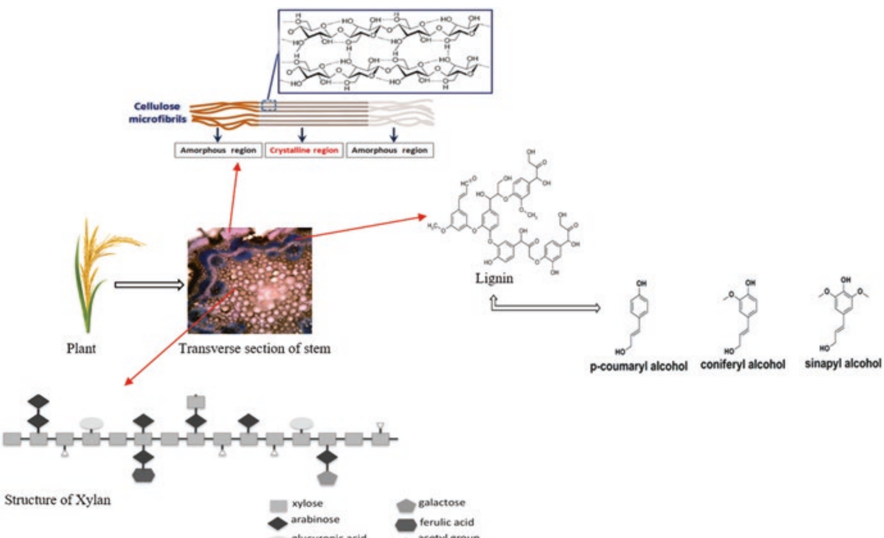

Fig. 16.1 Diagrammatic representation of the lignocellulosic structure: cellulose, hemicellulose and lignin

Most of the carbohydrate fraction is decomposed by microbial diversity in the ecological niches, releasing CO_2 and H_2O which is considered as a huge loss keeping in mind the potential of LCB to provide 2G ethanol and other value-added products (Chen 2014).

16.1.1 Cellulose

Cellulose, the most dominant structural moiety of plant cell walls, is a linear condensation polymer of D-glucose monomers joined together through β-1,4-glycosidic linkages. The molecular mass of cellulose is up to 2.4×10^6 g/mol, and the degree of polymerization (DP) varies from 100 to 20,000. The dimers of glucose (cellobiose) are the repeating subunits that constitute an organized fibrous structure. In nature, cellulose is present in both amorphous and crystalline forms. In its amorphous form, cellulose is found as unorganized branched polymeric chains and constitutes only a small percentage of the total cellulose. On the other hand, crystalline cellulose comprises of glucose molecules rotated at 180° with respect to the adjacent glucose units which further aggregate laterally through extensive hydrogen bonds and van der Waal's interactions to form well-organized and intertwined rod-like structures, known as microfibrils (Zhang and Lynd 2006; Fernandes et al. 2011). The crystalline cellulose exists in its two forms: cellulose 1α (triclinic) and cellulose 1β (monoclinic) with the latter being more predominant in plants. Further, six polymorphs of cellulose (I, II, III_I, III_{II}, IV_I and IV_{II}) are produced during pretreatment of the plant biomass. The cellulose I has been observed to be resistant to both chemical and enzymatic hydrolysis but it can be easily degraded upon transformation to cellulose III_I (Igarashi et al. 2007).

16.1.2 Hemicellulose

Hemicellulose is a heterogeneous polysaccharide made up of pentose and hexose sugars with xylan being the most abundant constituent (Uday et al. 2016; Luo et al. 2018). It is hydrophilic in nature and serves as a supporting material for the plant cell wall by cross-linking lignin and cellulose fibres (Vardakou et al. 2005). It has a complex structure with varying degrees of branching and consists of pyranoses (glucose and fructose), furanoses (arabinose and xylose) and uronic acids (D-glucuronic and D-galacturonic acids) linked together through β-(1,4)- or β-(1,3)-glycosidic bonds (Gírio et al. 2010). Xylan backbone is made up of β-1,4-linked xylose residues and is branched with different side groups. Depending on the nature of the substituents, xylans are categorized as arabinoxylans (α-L-arabinofuranosyl), glucuronoxylans (α-D-glucuronic acid and its 4-O-methyl ether derivative), glucurono arabinoxylan (α-D-glucuronic acid and α-L-arabinose) and galacto glucurono arabinoxylans (β-D-galactopyranosyl) (Motta et al. 2013; Burlacu et al. 2016). The degree of complexity increases from linearly substituted xylans to the highly substituted xylans. The nature of side chains determines topography, solubility and

interaction of the xylan molecules with other hemicellulosic constituents, therefore influencing the mode and extent of the enzymatic cleavage (Kulkarni et al. 1999). Glucomannan consists of a backbone of β-1,4-linked mannose and glucose monomers and is substituted with α-1,6-linked galactose (Gilbert et al. 2008). It has been observed that branched structure of hemicellulose makes it easily hydrolysable as compared to cellulose.

16.1.3 Lignin

Lignin is a phenolic macromolecule with complex structure and is present in the secondary thickened cell wall. It confers structural support, impermeability and resistance to the plant cell wall against microbial and enzymatic attack (Vanholme et al. 2010). It comprises of cross-linked polymers of three aromatic alcohol units: coniferyl, coumaryl and sinapyl alcohol in the form of guaiacyl (G), syringyl (S) and p-hydroxyphenyl (H) phenylpropanoid units, respectively. These monolignols are integrated into the lignin polymeric structure through alkyl-aryl, alkyl-alkyl and aryl-aryl ether bonds (Pu et al. 2008; Vanholme et al. 2010), and their composition varies depending upon different sources of lignin. Softwoods are rich in G (90% coniferyl alcohol), whereas angiosperms have a mixture of G and S (nearly equal amounts of coniferyl and sinapyl alcohol) and a significant proportion of p-coumaryl alcohol (10–20%) (Pu et al. 2008).

16.2 Pretreatment of Lignocellulosic Biomass

Bioconversion of LCB into 2G ethanol requires three important steps: pretreatment, hydrolysis and fermentation (Xiao et al. 2012). The lignocellulosic breakdown is obstructed by crystallinity of cellulose, degree of polymerization, degree of recalcitrance of lignin and several other physico-chemical factors (Yang and Wyman 2006; Kim and Lee 2006; Tian et al. 2018). Lignin is the major hindrance which restricts access of hydrolytic enzymes to the digestible parts of LCB. Also, lignin limits the rate of enzymatic hydrolysis through non-productive binding to the hydrolytic enzymes (Esteghlalian et al. 2001). Several pretreatment strategies have been adopted to disrupt the lignin-carbohydrate interactions and improve the overall hydrolysis yield.

The physical pretreatment strategies aim at reduction of particle size and crystallinity of the lignocellulosic substrates in order to reduce the degree of polymerization and enhance specific surface (Alvira et al. 2010; Taherzadeh and Karimi 2008). Several techniques like grinding, milling and chopping could be used depending upon the desired particle size of the substrate (Sun and Cheng 2002).

Steam explosion, the most widely employed physico-chemical pretreatment method, subjects LCB to pressurized steam for a few seconds to several minutes and then suddenly depressurizes. In the process, hemicellulose gets partially hydrolysed and solubilized, and the lignin is redistributed and removed to some extent. It exposes cellulose surface and increases accessibility of enzymes to the cellulose

microfibrils (Pan et al. 2005). This technique was commercially developed by Iogen Corporation (Canada) for degradation of hemicellulose and transformation of lignin due to high temperature; further, Beta Renewables Company (Italy) has adopted this technique for pretreatment of wheat straw and rice straw. POET-DSM and ANDRITZ (an international technology group) use corn cobs, leaves and husks treated with modified steam explosion technology for the production of renewable biofuel. Abengoa Bioenergy (Spain) prefers acid pretreatment followed by steam explosion for the production of 2G biofuel. The liquid hot water treatment is another chemical-free approach in which pressure is applied to maintain water at high temperature (160–240 °C) and provoke structural changes in the LCB. It solubilizes hemicellulose, increases accessibility of enzymes to cellulose and avoids formation of inhibitors (Mosier et al. 2005). The studies have shown that liquid hot water treatment significantly enhanced the hydrolysis of corn stover (Mosier et al. 2005), sugarcane bagasse (Laser et al. 2002) and wheat straw (Pérez et al. 2008). Ammonia fibre explosion (AFEX) involves treatment of biomass with liquid anhydrous ammonia at 60–100 °C and high pressure for different time intervals. During treatment, the release of pressure causes rapid expansion of ammonia gas which results in swelling and disruption of biomass fibres, partial decrystallization and distortion of lignin-carbohydrate interactions (Laureano-Perez et al. 2005). The dilute ammonia pretreatment at low temperature has been used by DuPont for bioconversion of corn stover and switch grass to bioethanol. Microwave (Keshwani 2009), ultrasound (Yachmenev et al. 2009) and CO_2 explosion (Schacht et al. 2008) are few other physico-chemical pretreatment techniques with very mild effects on the structure of biomass.

The chemical methods for the pretreatment of lignocellulosics focus on the use of acids, bases, oxidants and solvents. These are widely used in many pilot and demonstration plants as they are good for low lignin materials. In the acid pretreatment (acid hydrolysis), drilled and milled LCB is dipped in acidic solution at a specific temperature and pressure for a certain period of time. The pretreated unhydrolysed solid substrate is neutralized before subjecting it to enzymatic saccharification (Bensah and Mensah 2013; Rai et al. 2016a). The hydrolysis rate constant is directly proportional to the hydrogen ion concentration which implies that acids with more negative pKa value are more effective hydrolysing agents. However, dilute acid hydrolysis is more favoured at industrial scale keeping in view the problems associated with concentrated acids such as equipment corrosion, acid recovery and high operational and maintenance cost (Wyman 1996; Taherzadeh and Karimi 2008). Sulphuric acid (Saha et al. 2005) and hydrochloric, phosphoric and nitric acid (Mosier et al. 2005) have been tested for pretreatment of different LCBs with sulphuric acid being the most studied acid. There have been reports for saccharification yields as high as 74% when wheat straw was treated with 0.75% H_2SO_4 at 121 °C for 1 h (Saha et al. 2005). Another study on pretreatment of olive tree biomass with 1.4% H_2SO_4 at 210 °C shows 76.5% hydrolysis yield (Cara et al. 2008). Both HCl and HNO_3 possess good cellulose to sugar conversion rates, but their higher costs compared to H_2SO_4 limit their application. Ozone hydrolysis is also a powerful oxidant-based approach that has high delignification efficiency (Sun and Cheng 2002), but involvement of higher cost is again a limiting factor. The

Organosolv process developed by Lignol in integration with the University of British Columbia uses organic solvents such as alcohols (methanol, ethanol, tetrahydrofurfuryl alcohol) and organic acids to treat LCB (Zhao et al. 2009; Mesa et al. 2011). The use of ionic liquids (ILs) has received great attention in the recent years as it offers a potentially greener alternative to the organic solvents for lignocellulosic pretreatment (Hayes 2009; Socha et al. 2014).

Although alkaline pretreatment strategies have not been reported beyond lab scale, these are quite efficient in increasing the enzyme effectiveness by eliminating non-productive adsorption sites through lignin removal (Kumar et al. 2009). These strategies use sodium hydroxide (Modenbach 2013), ammonia (Kim et al. 2003; Kim and Lee 2007; Yoo et al. 2011) and calcium hydroxide (Kaar and Holtzapple 2000) as chemical catalysts. NaOH attacks ether and ester bonds in lignin-carbohydrate complexes as well as ester and carbon-carbon (C-C) bonds in the lignin molecules. During pretreatment, NaOH dissociates into Na^+ (sodium) and OH^- (hydroxide) ions, and the concentration of OH^- ions determines the rate of hydrolysis. The ammonia treatment leads to removal of lignin, increase in surface area and opening up of the lignocellulosic structure, making it amenable to enzymatic attack. The calcium hydroxide method is used especially for treatment of herbaceous biomass (grasses) through removal of acetyl groups and lignin (~30%).

The biological pretreatment methods rely upon brown, white and soft-rot fungi which are capable of degrading lignin, hemicellulose and very little of cellulose (Sánchez 2009). Lignin degradation using white rot fungi is the most efficient biological method for pretreatment of LCB and it occurs through the action of lignin-degrading enzymes such lignin peroxidase, manganese peroxidase and laccase (Shi et al. 2008; Kumar et al. 2009). A study showed that pretreatment of wheat straw with fungal isolate RCK-1 for 10 days resulted in reduced acid loading for hydrolysis, increased yield of fermentable sugars and reduced concentration of fermentation inhibitors (Kuhar et al. 2008). In a report from Singh et al. (2008), pretreatment of sugarcane trash employing eight different bacterial and fungal strains significantly enhanced the accessibility of sugars for enzymatic hydrolysis. The biological methods offer several advantages such as low energy requirement, low capital cost, no chemical requirement and mild operating conditions; however, low rate of hydrolysis compared to other pretreatment technologies is the limiting factor (Sun and Cheng 2002). Conclusively, there is no ideal method that could be generalized for pretreatment of all the substrates; therefore, the choice of methods will depend greatly on the composition and recalcitrant nature of the LCB.

16.3 Diverse Lignocellulolytic Glycosyl Hydrolases and Auxiliary Enzymes Produced by Microbes

The enzymes responsible for synthesis, modification and degradation of carbohydrates are clustered as carbohydrate-active enzymes (CAZymes). These enzymes are classified into glycosyl hydrolases (GH), polysaccharide lyases (PL), carbohydrate esterases (CE) and glycosyl transferases (GT) on the basis of their similarity

to amino acid sequences, enzymatic mechanism and protein folding (Levasseur et al. 2013). The efficient bioconversion of LCB into fermentable sugars (pentoses/hexoses) is achieved through an array of enzymes collectively known as glycosyl hydrolases (GH). These enzymes are responsible for hydrolysing glycosidic bonds between two sugars or a sugar and one non-sugar moiety within oligosaccharides or carbohydrates (Ezeilo et al. 2017). A total of 115 GH families based on their mode of action and amino acid sequence have been identified till date. Despite the vast diversity of GHs, their active site topology remains the same and can be classified into three categories: pocket, cleft and tunnel (Davies and Henrissat 1995). The enzymatic cleavage of glycosidic linkages is achieved through two major mechanisms resulting in an overall inversion or retention of the anomeric configuration (Koshland 1953; Sinnott 1990; Davies and Henrissat 1995). In the inverting mechanism, protonation of the glycosidic oxygen and departure of aglycone occurs through a concomitant attack of an activated water molecule (activated by a base residue). This single nucleophilic substitution reaction results in a product with stereochemistry opposite to the substrate. Alternatively, the retention mechanism operates through two steps: glycosylation and deglycosylation. In the first step, protonation of glycosidic oxygen is mediated by an acid catalyst and a base provides the nucleophilic assistance to aglycone departure. In the second step, the resulting glycosyl enzyme is subjected to hydrolysis by a water molecule through nucleophilic substitution at an anomeric carbon. This generates a product with stereochemistry identical to that of the substrate (Dodd and Cann 2009; Vuong and Wilson 2010).

The sites for binding sugar residues in glycosyl hydrolases are known as subsites and the glycosidic bond cleavage within sugar residues occurs either at −1 subsite (non-reducing) and +1 subsite (reducing end) (Davies et al. 1997).

16.3.1 Carbohydrate-Binding Modules

Carbohydrate-binding modules (CBMs) are defined as the adjacent amino acid sequences within polysaccharide-degrading enzymes with discrete folding pattern and having carbohydrate-binding activity (Boraston et al. 2004). GHs are unable to hydrolyse insoluble polysaccharides because of their inaccessibility to specific positions of the substrate. In order to overcome this problem, GHs are equipped with CBMs. In most of the enzymes, a catalytic domain (CD) is connected to CBM through a linker segment that is rich in Pro/Ser/Thr (Hasper et al. 2002). The length of the linker varies within different GH families and has been reported to have a significant influence on the enzyme activity as well as binding affinity. The eukaryotic, bacterial and archaeal linkers are 250, 86 and 73 amino acids long, respectively (Wang et al. 2011). It has been observed that shorter linker length promotes processivity in *T. reesei* Cel7A, whereas longer linker lengths in Cel6A promote search for hydrolytic sites (Beckham et al. 2010; Sammond et al. 2012; Lima et al. 2013). The CAZy database has grouped CBMs into 81 different families (based on amino acid sequence similarity); out of which, 54 families have been classified into Type A, B

and C CBMs (Boraston et al. 2004; Lombard et al. 2013; Ezeilo et al. 2017). Type A CBMs are the most distinct which bind specifically to the surface of insoluble, highly crystalline polysaccharides such as cellulose and chitin (McLean et al. 2000). Type B CBMs are glycan chain-binding modules and are often appended with enzymes such as cellulases, xylanases and mannases. Type C are a unique class of CBMs having a 'lectin-like' feature, and they bind optimally to mono-, di- or trisaccharides (small sugars) [Boraston et al. 2004].

16.3.2 Cellulosomes

Cellulosomes are the multienzyme assemblages produced by anaerobic bacteria and fungi such as *Clostridium*, *Acetovibrio*, *Ruminococcus*, *Bacteroides*, *Nocallimastix*, *Orpinomyces* and *Piromyces* (Doi and Kosugi 2004; Fontes and Gilbert 2010; Blumer-Schuette et al. 2014). A typical cellulosome consists of a large backbone scaffolding protein which contains several cohesins (tandem modules). The active protein components adhere to cohesins through their dockerin modules (Noach et al. 2010; Hamberg et al. 2014; Smith et al. 2017). These nano-machines assemble key lignocellulolytic enzymes along with many auxiliary modules such as CBMs and other types of proteins and enzymes (Blouzard et al. 2010). Although the structure-function relationship of the cellulosomes is not completely understood, the studies on purified and synthetically assembled cellulosomes have shown that their catalytic efficiency for hydrolysis of natural complex cellulosic substrates (containing lignin) is significantly higher as compared to the enzyme cocktails of the same composition (Fierobe et al. 2005; Fendri et al. 2009; Arfi et al. 2014). This synergism may attribute to the higher-order structure of cellulosomes (Vazana et al. 2012; Stern et al. 2016). The classical methods for assessing structure of cellulosomes are not apt because of their heterogeneous, large and sophisticated construct containing oligosaccharides, flexible linkers and regions of intrinsic disorder (Bayer et al. 2009; Molinier et al. 2011). However, the 'dissect and reconstruct' strategies employed in concert with structural biology approaches have revealed partial, 3D arrangements of the cellulosomes in a recent study by Smith and Bayer (2013).

16.3.3 Cellulases

Cellulases are the active members of GH group of CAZymes that catalyse hydrolysis of cellulose to glucose through cleavage of β-1,4-glycosidic linkages (Lynd et al. 2002; Mba Medie et al. 2012). The cellulose degradation is accomplished through three major groups of enzymes: endoglucanases (E.C.3.2.1.4; EG), exoglucanases (E.C.3.2.1.74 and E.C.3.2.1.91; CBH) and β-glucosidase (E.C.3.2.1.21; βG) (Zhang and Lynd 2004; Zhang et al. 2006). Endoglucanase (EG) randomly hydrolyses β-1,4-glycosidic linkages of the amorphous cellulose, releasing oligomers (such as cellobiose, cellotriose, cellotetraose) with exposed reducing and non-reducing ends. EGs are broadly classified into GH families 5, 6, 7, 8, 9, 12, 44, 45, 48, 51, 74 and

124, and most of these possess cleft-shaped active sites (Sweeney and Xu 2012; Ezeilo et al. 2017). The crystallographic studies of the enzyme belonging to GH families 5, 6, 7, 8, 9, 45 and 48 have revealed variations in their folding topologies (Bourne and Henrissat 2001). The enzymes belonging to GH 5 and 7 follow retention mechanism, whereas those belonging to GH 6, 9 and 48 proceed through inverting mechanism (Mingardon et al. 2007; Miotto et al. 2014). EGs belonging to GH 5 and 9 act processively (Zhang et al. 2014) with the reports of bifunctional processive EGs (endo- and exo-type activities) from a brown rot basidiomycete *Gleophyllum trabeum* (Cohen et al. 2005) and a marine bacterium *Saccharophagus degradans* 2-40 (Watson et al. 2009). Further, CBMs are found to play a critical role in catalysing hydrolysis of cellulose by processive EGs belonging to GH 9; however, an exception of this has been reported from Cel9A of *Cytophaga hutchensonii* which lacks CBM. The loss in processivity and cellulose degradation ability of CelE4 (GH9) was observed when the enzyme was subjected to CBM3c deletion (Irwin et al. 1998; Li et al. 2007).

The exoglucanase takes up the released oligosaccharides and act processively either from reducing (CBHI) or non-reducing (CBHII) termini to yield cellobiose (CBH) and/or glucose (glucohydrolase). CBH enzymes belong to GH families 5, 6, 7, 9, 48 and 74 (Poidevin et al. 2013). The fungal CBHs are found in GH 6 and 7, and GH 6 constitutes aerobic bacterial CBHs, whereas GH 48 contains CBHs from anaerobic fungus and bacteria. The structural and functional analysis shows that CBHs from ascomycetes are comprised of four loops that offer stronger binding affinity and enhanced processivity by covering the active subsite. The tunnel-like active sites make these enzymes proficient in degrading crystalline cellulose processively. These features confer deviation to CBH kinetics from Michaelis-Menten model and exhibit a significantly different pattern and local jamming effect (Xu and Ding 2007; Igarashi et al. 2011). On the other hand, CBHs from basidiomycetes lack one of the four loops covering the active subsite.

β-Glucosidases (βGs) are the third group of cellulases that catalyse hydrolysis of cellobiose or short-chain oligosaccharides (up to cellohexaose) to form glucose and relieve end product inhibition of EGs. Based on their substrate specificity, βGs can be categorized as (a) enzymes with high substrate specificity towards aryl β-D-glucosides, (b) enzymes that preferentially hydrolyse cellobiose and cellobiases and (c) enzymes specific to both types of substrates. The catalytic core of βG possesses pocket-shaped active site, a topology that pushes the enzyme to act on non-reducing ends of its substrate (Decker et al. 2000). βG enzymes belong to GH 1, 3, 5, 9, 30 and 116 and play an important role in the efficient hydrolysis of cellulosic fraction of LCB into glucose that could be subsequently fermented into 2G ethanol; however, the rate of hydrolysis is inversely proportional to the DP of the substrate (Ezeilo et al. 2017). It has been observed that most of the fungal βGs reported so far belong to GH 3, one of the most abundant family in CAZy database comprising more than 6000 enzymes distributed among plants, fungi and bacteria (Bhatia et al. 2002; Singhania et al. 2013). Apart from the βG activity, enzymes belonging to GH 3 family also exhibit β-D-xylosidase, α-L-arabinofuranosidase and N-acetyl-β-D-glucosaminidase activities (Faure 2002; Harvey et al. 2000).

16.3.4 Hemicellulases

Hemicellulases are the diverse group (GHs) of enzymes that catalyse depolymerization of hemicellulose through synergistic action of endoxylanase (E.C.3.2.1.8), β-xylosidase (E.C.3.2.1.37), α-glucuronidase (E.C.3.2.1.139), α-arabinofuranosidase (E.C.3.2.1.55), arabinose (E.C.3.2.1.99), acetyl xylan esterase (E.C.3.1.1.72) and feruloyl xylan esterase (E.C.3.1.1.73) (Kumar et al. 2008; Juturu and Wu 2013). Among all the hemicellulases, xylanases (endo-xylanase and β-xylosidase, collectively) have been extensively reviewed attributing to the fact that xylan is the major constituent of hemicelluloses. Endo-xylanases catalyse the cleavage of β-xylosidic bonds that hold together 2-D-xylopyranosyl residues in the xylan backbone. Majority of the identified endoxlyanases belong to GH 5, 8, 10, 11 and 43 where the members of GH 5, 10 and 11 exhibit retaining mechanism and those from GH 8 and 43 operate through inverting mechanism (Cantarel et al. 2008; Lombard et al. 2013). GH 10 and 11 are the most studied endoxylanases with GH 10 endoxylanases having broad substrate specificity. GH 10 are the high molecular weight plant, fungal and bacterial enzymes with a catalytic domain (CD) and a $(\beta/\alpha)_8$-fold barrel CBM. They have four to eight substrate binding sites and an active site that is located in a deep groove of the β-strand at the C-terminal side. They are capable of hydrolysing linear and substituted heteroxylans, xylo-oligosaccharides and aryl-cellobiosides, but not cellulose. In contrast, GH 11 consists of true endoxylanases that cleave β-1,4-glycosidic bonds of heteroxylans and hydrolyse them into xylobiose and xylotriose (Katapodis et al. 2003; Vardakou et al. 2008). These are low molecular weight enzymes consisting of one β-jelly-roll-shaped catalytic domain composed of two glutamates which act as catalytic residues (Dodd and Cann 2009; Paës et al. 2012).

β-Xylosidases belong to GH families 3, 39, 43, 52 and 54 and play an important role in hydrolysing β-1,4 bonds in xylobiose and other short oligosaccharides and relieving end product inhibition of endoxylanases (Shallom and Shoham 2003; Knob and Carmona 2009). β-Xylosidases from yeast and bacteria are usually cell bound (in cytosol), whereas fungal β-xylosidases are released into a medium at later stages of growth either by true secretion or as a result of cell lysis (Wong and Saddler 1992; Bajpai 1997). The fungal β-xylosidases have been reported from GH 3, 43 and 54 where members of GH 3 and 54 families follow retention mechanism and GH 43 enzymes operate through an inverting mechanism. GH 3 β-xylosidase have Glu and Asp as their putative catalytic residues which remain conserved within all the members of GH family 3. A GH 43 β-xylosidase from *Selenomonas ruminantium* was reported to possess an N-terminal five bladed β-propeller domain and a C-terminal α/β sandwich domain (Brunzelle et al. 2008). Another study on GH 43 β-xylosidase from *Thermobifida fusca* revealed that both the modules are necessary for the activity of the enzyme (Moraïs et al. 2012). The spatial similarity between the structure of D-xylopyranose and L-arabinofuranose results in xylosidase-arabinosidase dual activities. These bifunctional enzymes have been reported from GH families 3, 43 and 54 (Mai et al. 2000; Lee et al. 2003).

α-L-Arabinofuranosidase and α-L-arabinanase belonging to GH 3, 43, 51, 54 and 62 are known to play an important role in hydrolysing arabinofuranosyl-containing hemicelluloses (Shallom and Shoham 2003). Some of these enzymes exhibit activity against a broad spectrum of substrates. They act on arabinofuranoside moieties at *O*-5, *O*-2 and/or *O*-3 as a single substituent as well as from *O*-2 and *O*-3 doubly substituted xylans, xylooligomers and arabinans (Saha 2000). The crystal structure of α-L-arabinanase (GH 43) from *Cellvibrio japonicus* revealed a five-bladed β-propeller fold (Nurizzo et al. 2002).

β-Mannanases and β-mannosidases are involved in the hydrolysis of mannan-based hemicelluloses into short β-1,4-manno-oligomers and subsequently into mannose. β-mannanases are found in GH families 5 and 26, whereas β-mannosidases belong to GH families 1, 2 and 5 (Shallom and Shoham 2003). The crystal structure of a GH 26 β-mannanase from *C. japonicus* revealed a $(\beta/\alpha)_8$-fold structure with two catalytic residues located at the ends of β strands 4 and 7 (Hogg et al. 2001).

The hemicellulolytic esterases include acetyl xylan esterases (AXEs) and feruloyl xylan esterases (FAEs), where the former hydrolyses acetyl substitutions on xylose moieties and the latter catalyses hydrolysis of ester bonds between arabinose substitutions and ferulic acid (Ulaganathan et al. 2015). These ester bonds are involved in cross-linking xylan to lignin (Shallom and Shoham 2003). An intracellular Axe2 (*G. stearothermophilus*) belonging to lipase GDSL family was characterized to possess a 'doughnut-shaped' homo-octameric structure where active sites (8) were arranged in tightly packed pairs (4), facing the internal cavity (Lansky et al. 2014). A single domain FAE (Tx-Est 1) containing conserved catalytic triad (putative lipase) residues Ser-Asp-His in its C-terminal part has been characterized from *Thermobacillus xylanilyticus* (Rakotoarivonina et al. 2011).

16.3.5 Auxiliary Enzymes

A new paradigm for degradation of cellulose has focused on a recently discovered oxidative system in which extracellular cellobiose dehydrogenase (CDH) act synergistically with the copper-dependent lytic polysaccharide monoxygenases (GH61; AA9; LPMOs) (Dumonceaux et al. 2001; Vaaje-Kolstad et al. 2010; Canam et al. 2011). This oxidative system catalyses redox-mediated cleavage of glycosidic bonds in crystalline cellulose, hemicellulose and starch (Horn et al. 2012; Agger et al. 2014; Vu et al. 2014; Müller et al. 2017; Chylenski et al. 2017). The fungal genomes harbour many of the genes including LPMOs which require detailed functional characterization. LPMOs are widespread among the fungal genera where *N. crassa* (Phillips et al. 2011), *Thielavia terrestris* (Merino and Cherry 2007) and *Myceliophthora thermophila* (Frommhagen et al. 2016) have been identified as the prolific producers of the enzyme. Quinlan et al. (2011) carried out structural characterization of GH61 (*T. auranticus*) and published the first report for copper dependence of these enzymes. This property was further validated by two other research groups in their respective studies on GH61 derived from *N. crassa* and *P. chrysosporium* (Phillips et al. 2011; Westereng et al. 2011). LPMOs facilitate direct

hydroxylation of the crystalline polysaccharide substrates at C1 or C4 producing aldonic acid or 4-Keto sugars, respectively (Beeson et al. 2011; Quinlan et al. 2011; Tan et al. 2015). Although the precise mechanism of monoxygenation reaction is not well understood, superoxo and oxyl mechanisms are likely to be involved in the substrate hydroxylation (Beeson et al. 2011; Phillips et al. 2011; Li et al. 2012; Kim et al. 2014). These enzymes require an electron donor to kick-start their activity, and CDHs occur as their natural companion (electron donors). CDH is a large flavocytochrome that contains a haem b binding cytochrome domain (CYT) connected to a flavin adenine dinucleotide (FAD) binding dehydrogenase domain (DH) through a long, flexible linker (Zamocky et al. 2006). CDHs are classified as Class I and Class II CDHs where Class I CDHs occur in basidiomycetes and lack additional domains. On the other hand, Class II CDHs are produced by ascomycetes and are subcategorized into Class IIA and Class IIB corresponding to the presence and absence of Type 1 CBMs, respectively (Zamocky et al. 2006; Harreither et al. 2011). The Class I CDHs bind strongly to the cellulose surface even in the absence of CBMs; however, the mechanism of binding remains unknown (Henriksson et al. 1995; Tan et al. 2015). The DH domain oxidizes cellobiose (C1) to cellobiono-1,5-lactone with the reduction of FAD followed by inter-domain electron transfer (IET) from the reduced FAD to cyt haem b. The single electron transfer (ET) follows the transfer of electron from CYT to the external electron acceptors such as LPMOs (Zamocky et al. 2006). The studies on laccase-mediated oxidation of phenolic and non-phenolic components of lignin (Bourbonnais and Paice 1990; Arora and Sharma 2010; Chen et al. 2016) have suggested that laccases could also play a significant role in the degradation of lignocellulosic biomass. Laccases are the multi-copper oxidases having dynamic potential to oxidize a broad spectrum of inorganic and organic compounds like phenols, polyphenols, anilines etc., through one electron transfer mechanism (Messerschmidt and Huber 1990; Kunamneni et al. 2008). However, oxidation of non-phenolic moieties of lignin requires co-presence of primary laccase substrates such as 2,2′-azino-bis-[3-ethylthiazoline-6-sulfonate] (ABTS) that act as redox mediators. These enzymes consist of at least four copper ions (referred to as T1, T2, T3α and T3β) that are generally incorporated in the enzyme active site (Kunamneni et al. 2008).

16.4 Lignocellulolytic Microorganisms from Diverse Habitats

The microorganisms capable of producing cellulases, hemicellulases and auxiliary enzymes inhabit a diverse range of ecological niches such as decaying plant materials, soil, compost piles, rumens, forest waste piles, wood processing plants and sewage sludge (Amore et al. 2013). Although fungi are the major role players in degradation of plant biomass in nature, cellulolytic bacteria also exhibit some unique properties which provide a variety of lignocellulolytic enzymes for biofuel and biorefining industry (Maki et al. 2009; Pang et al. 2017). Table 16.1 shows some recently reported cellulolytic/hemicellulolytic microbial strains. Several cellulolytic bacteria isolated from different environments include anaerobic bacteria like

Table 16.1 Cellulases and hemicellulases produced by different microorganisms

Organism	Enzymes	Folding topology	Mechanism	References
Clostridium cellulolyticum	EG, CBH	$(\alpha/\alpha)_6$	Inverting	Mandelman et al. (2003)
Caldicellulosiruptor bescii	β-galactosidase, βG	$(\alpha/\beta)_8$-Tim barrel	Retaining	Bai et al. (2013)
Acrophialophora sp.	EG, CBH, βG, Xyl	–	–	Rai et al. (2016a)
Melanocarpus albomyces	EG	Six-stranded β-barrel	Inverting	Hirvonene and Papageorgiou (2003)
Penicillium brasilianum IBT 20888	EG, βG, Xyl, β-xylo, α-galacto, Afase	–	–	Jørgensen and Olsson (2006)
Humicola grisea	EG	β-jelly roll	Retaining	Li et al. (2011)
Malbranchea cinnamomea strain S 168	Xyl	–	–	Fan et al. (2014)
Thermoascus auranticus	EG, CBH, Xyl, β-mannosidase, β-mannanase	$(\beta/\alpha)_8$	Retaining	Li et al. (2011)
Scytalidium thermophilum ATCC No. 16454	Xyl	–	–	Kocabaş et al. (2015)
Humicola insolens	EG, CBH	α-/β-barrel	Inverting	Bu et al. (2012)
Remersonia thermophila CBS 540.69	Xyl	–	–	McPhillips et al. (2014)
Trichoderma reesei	EG, CBH	β-jelly roll	Retaining	Shibafuji et al. (2014)
Penicillium decumbens 114-2	EG, CBH, βG	–	–	Sun et al. (2008)
Penicillium oxalicum GZ 2	EG, CBH, βG, Xyl	–	–	Liao et al. (2014)
Penicillium sp. Dal 5	EG, CBH, βG, Xyl	–	–	Rai et al. (2016b)

Bacteroides, *Acetivibrio*, *Ruminococcus* and *Clostridium* and aerobic bacteria such as *Microbispora*, *Cellulomonas*, *Cellvibrio*, *Thermobispora*, *Bacillus* sp., *Thermomonospora* sp., *Pseudomonas* sp., *Nocardiopsis* sp., *Erwinia chrysanthemi*, *Streptomyces* spp. and *Thermobifida fusca* (Sadhu and Maiti 2013; Pang et al. 2017). *C. phytofermentans* was reported by Warnick et al. (2002) for the production of an endoglucanase capable of hydrolysing both crystalline cellulose and soluble carboxymethyl cellulose (CMC). An endoglucanase with chitonase activity was isolated and cloned from *Paenibacillus cookie* (Shinoda et al. 2012). Aerobic bacteria such as *Pseudomonas cellulosa* and *Bacillus stearothermophilus* produce restricted number of hemicellulases resulting in large oligomers which are decomposed into monomers by the intracellular enzymes in the second step of hydrolysis (Knob et al.

2010). *Geobacillus* sp. WSUCF1 has been reported for the production of highly thermostable endoxylanase (GH 10) and β-xylosidase (GH 39) (Bhalla et al. 2014a; Bhalla et al. 2014b). Some additional bacterial strains such as *Streptomyces* sp., *Arthrobacter* sp. and *Geobacillus stearothermophilus* have also been reported for production of hemicellulases. Novel bacteria from diverse ecological niches such as methanogenic sludge (*Bacteroides luti*) and surface of seashore (*Oricola cellulosilytica*) have been reported (Shinoda et al. 2012; Hameed et al. 2015). *Caldicellulosiruptor bescii* is the most studied bacterial strain known for its ability to produce multifunctional endoglucanase with catalytic domain for GH5 and GH45 (Bai et al. 2013).

Lignocellulolytic fungi are the most suited candidates for degradation of cellulose and hemicellulose as they are known to produce copious amounts of these extracellular enzymes in their secreted form (Phitsuwan et al. 2013). *Chaetomium thermophilum*, *Humicola insolens*, *H. grisea*, *Myceliophthora thermophila*, *Talaromyces emersonii*, *Thermoascus auranticus*, *Sporotrichum thermophila* and *Melanocarpus albomyces* are the thermophilic lignocellulolytic fungal strains reported from diverse ecological niches (Jatinder et al. 2006; Zambare et al. 2011). Among different fungal strains, *Trichoderma reesei* is the most widely exploited expression platform for the production of cellulases (Gusakov 2011). The GH mixtures originated from *Aspergillus* sp. have also been exploited commercially (Marjamaa et al. 2013). The *Aspergillus* enzyme cocktails exhibit high β-glucosidase activity, but their endoglucanase activity is low, whereas *Trichoderma* strains usually exhibit high endo- and exoglucanase activities, but their expression of β-glucosidase is low, and therefore, efficacy of both the enzyme mixtures for hydrolysis of cellulose is also quite low (Kumar et al. 2008). In this context, novel and more efficient lignocellulolytic GHs are required from alternate microbial sources in order to attain better hydrolysis at low protein and high substrate loadings. Many other fungal strains such as *Penicillium decumbens*, *Acremonium cellulolyticus* and *M. thermophila* are capable of producing cellulolytic/hemicellulolytic enzymes and have been employed for commercial-level enzyme production in China, Japan and Dyadic industries (Fujii et al. 2009; Gusakov 2011; Liu et al. 2013c). Moreover, recent studies have shown that cellulases from many *Penicillium* strains show better catalytic and hydrolytic potential when compared to *T. reesei* at equal protein loadings (Chekushina et al. 2013; Marjamaa et al. 2013). *P. echinulatum* (Sehnem et al. 2006; Martins et al. 2008), *P. brasiliuanum* (Jørgensen et al. 2003; Jørgensen and Olsson 2006), *P. decumbens* (Sun et al. 2008), *P. funiculosum* (Adney et al. 2008; Guais et al. 2008), *P. purpurogenum* (Lee et al. 2010) and *P. pinophilum* (Wood et al. 1989; Singh et al. 2009b; Joo et al. 2010) are the different *Penicillium* strains reported for the production of highly efficient cellulolytic enzymes. Although knowledge on the enzyme systems from *Penicillium* strains is established, their composition and regulation are scarce in comparison to *T. reesei* and *Aspergillus* strains, but some of the *Penicillium* strains such as *P. funiculosum* have been utilized for commercial production of cellulases (Guais et al. 2008). In addition, the potential of enzymatic machineries from *P. pinophilum* (Singh et al. 2009b), *P. janthinellum* EMS-UV-8 and *P. funiculosum* (Saini et al. 2016), *P. oxalicum* EU2106 (Huang

et al. 2015) and *Penicillium* sp. Dal5 (Rai et al. 2016b) has been evaluated and validated for the efficient hydrolysis of differently pretreated LCB. The long-term strain improvement of *P. decumbens* 114 through repeated mutagenesis resulted in a developed strain (JU-A10-T) with hyperactivity of cellulases and hemicellulases (Liu et al. 2013b). A trigenic recombinant strain 'RE-10' developed from *P. oxalicum* 114-2 through deletion of *bgl2* and *creA* along with overexpression of *clrB* gene showed improved cellulolytic ability and increased filter paper and extracellular protein concentration by up to 20 and 10 folds, respectively (Yao et al. 2015).

16.5 Secretome Analysis to Study Diversity of Glycosyl Hydrolases and Auxiliary Enzymes

The term secretome refers to proteins secreted by microbial cells through classical, non-classical or exosomal pathways (Pavlou and Dimandis 2010). Recent advances in chromatographic and spectrophotometric techniques along with the availability of annotated genomes and sophisticated bioinformatics tools have made expression profiling of proteins feasible (Bouws et al. 2008). This comprehensive approach has been widely used for describing fungal secretomes, expression profiling and identification of new enzymes (Sato et al. 2007; Kim et al. 2008). Marx et al. (2013) utilized this approach to study diverse profiles of polysaccharide-degrading hydrolases in fungal secretome under the influence of different carbon and nitrogen sources. Kaur et al. (2013) followed this approach to reveal differential expression of proteins in the parent and developed heterokaryon. LC-MS/MS-based investigation of the secretome derived from commercial *T. reesei* strains revealed CBHI (Cel7A) as the most predominant protein followed by CBHII (Cel6A) (Chundawat et al. 2011). The secretome analysis of *T. harzianum* revealed that majority of chitinases and endochitinases in addition to other hydrolytic enzymes are produced under the influence of cellulose (do Vale et al. 2012). Ravalason et al. (2008) performed proteomic analysis to determine compositional differences in the secretome of *Phanerochaete chrysosporium* CIRM-BRFM41 when grown under ligninolytic conditions and on soft wood chips under biopulping conditions. LC-MS/MS-based analysis of the secretomes obtained from *A. fumigatus* Z5 in the presence of glucose, avicel and rice straw revealed that most of the lignocellulolytic GHs were upregulated when rice straw and avicel were used as carbon sources (Liu et al. 2013a). The secretome analysis of *M. cinnamomea* revealed the production of a spectrum of GHs and other lytic polysaccharides produced using sorghum straw as carbon source (Mahajan et al. 2016). LTQ-Velos-Oritrap mass spectrometry analysis of *Acrophialophora* sp. P2 secretome could unveil the identity of only 26 proteins attributing to the scarce *Acrophialophora* protein database (Rai et al. 2016a). The mass spectroscopy analysis of *Penicillium* sp. Dal 5 secretome identified a total of 108 proteins containing an array of GH, PL, CE, LPMOs and swollenin which makes it a good enzymatic machinery for hydrolysis of lignocellulosics (Rai et al. 2016b). The secretome studies conducted by Liu et al. (2013c) revealed that *P. decumbens* has a more diverse lignocellulolytic enzyme machinery than *T. reesei*.

Moreover, the production medium supplemented with cellulose and wheat bran induced significantly higher expression of these proteins in *P. decumbens* secretome as compared to glucose. The analysis of secretome produced by *P. oxalicum* GZ-2 showed that the addition of xylan to cellulose medium resulted in higher expression of hemicellulases and strong induction of cellulases (Liao et al. 2014). The comparative analysis of *A. niger*, *T. reesei* and *P. oxalicum* 114-2 revealed that cellulase system produced by *P. oxalicum* 114-2 is more balanced with diversity of enzymes involved in degradation of xylan and β-D-glucans (Gong et al. 2015). The proteomic analysis of three commercial cellulase preparations, i.e. SP from *P. oxalicum* JU-A10T, ST from *T. reesei* SN1 and Celluclast 1.5 L from Novozyme, disclosed that both SP and ST are rich in carbohydrate-degrading enzymes and multiple non-hydrolytic proteins with greater number of CBM1 in SP (Song et al. 2016). A comprehensive insight into the secretome of a hyper-cellulolytic *P. funiculosum* was provided by LC-MS/MS analysis performed by Ogunmolu et al. (2015). The secretome analysis of *P. purpurogenum* showed the presence of multienzyme complexes under non-denaturing conditions (Gonzalez-Vogel et al. 2011). Further, recent introduction of iTRAQ (isobaric tags for relative and absolute quantification) technique has eliminated the limitations of 2D gel electrophoresis and improved the throughput of proteomic studies (Adav et al. 2010; Liu et al. 2014).

16.6 Characteristics of Novel Lignocellulolytic Enzymes from Diverse Microorganisms

Although secretome analysis is now widely employed to unveil GH composition of the protein cocktails, the detailed functional characterization of proteins still requires purification. Several chromatographic techniques such as ion exchange, size exclusion, chromatofocussing and hydrophobic interaction chromatography are being used to remove contaminating proteins present in the secretome and purify the protein of interest. There are several reports on purification and characterization of cellulases and hemicellulases from different microbial sources. Oyekola et al. (2007) purified two endoglucanases (66.2 and 52.4 kDa) from an anaerobic sulphidogenic bioreactor through ion exchange chromatography. Several aerobic thermophilic bacteria such as *Acidothermus cellulolyticus* (Sakon et al. 1996), *Rhodothermus marinus* (Hreggvidsson et al. 1996), *T. fusca*, *Thermatoga maritima* (Evans et al. 2000), *Caldibacillus cellulovorans* (Bergquist et al. 1999) and *Paenibacillus* sp. (Ogawa et al. 2007) have been reported for the purification of endoglucanases. A thermostable EG with temperature optimum of 70 °C was purified from *T. aurantiacus* (Dave et al. 2015). Two cellulases Cel12A and Cel45A showing affinity towards xyloglucan in addition to CMC and barley β-glucan have been purified from *C. lucknowense* (Bukhtojarov et al. 2004). An extracellular βG purified from *A. niger* Au0847 exhibited $t_{1/2}$ of 15 min at 70 °C as well as great potential for hydrolysing geniposide to genipin (Gong et al. 2014). Another βG active at 60 °C with high affinity towards salicin was purified from *A. glaucus* (Ma et al. 2011). The characterized enzyme was similar to βGs reported from *A. japonicas* (Decker et al. 2000),

P. pinophilum (Joo et al. 2010) and *H. insolens* (Souza et al. 2010). *T. reesei* QM9414 secretome showed enhanced saccharification of cellulose upon supplementation with a βG purified from *P. decumbens* (Chen et al. 2010). CBHI purified from industrial cellulolytic strains *T. reesei* and *P. decumbens* were found to be optimally active at 50 °C (Payne et al. 2015). CBHI from *Acrophialophora* sp. P2 showed optimum activity 60 °C, pH 5.0 and 6.0 equally (Rai et al. 2016a). Two xylanases GH10 (30 kDa) and GH11 (25.2 kDa) were purified and characterized from *M. flava* (Sharma et al. 2010).

A GH30 xylanase (XYN IV) exhibiting both exo- and endoxylanase activities has been reported from *T. reesei* (Tenkanen et al. 2013). An acidophilic endoxylanase has recently been purified from *P. oxalicum* (Liao et al. 2014). An endoxylanase (18.36 kDa) showing temperature and pH optimum of 60 °C and 3.0, respectively, was purified from *P. glabrum* (Knob et al. 2013). A novel thermostable endoxylanase (GH10) cloned from bacterium *Geobacillus* s. WSUCF1 was found to retain 82%, 60% and 50% of its original activity at 50, 55 and 60 °C, respectively, after 60 h (Bhalla et al. 2014a). Fritz et al. (2008) purified three α-L- arabinofuranosidases (Afase) from *P. purpurogenum.* A GH54 Afase specific for maize fibre, oat spelt arabinoxylan and wheat arabinoxylan was purified and characterized from *Aurobasidium pullulans* (De Wet et al. 2008). Another Afase belonging to GH 43 has been reported from *P. chrysogenum* for its strong activity on sugarbeet (Shinozaki et al. 2014). A β-xylosidase (97 kDa) with optimum activity at 50 °C and pH 7.0 was purified and characterized from *T. thermophilus* (Guerfali et al. 2008). A highly thermostable β-xylosidase with $t_{1/2}$ of 9 days at 70 °C has been reported from *Geobacillus* sp. WSUCF1 (Bhalla et al. 2014b). Ravanal et al. (2013) reported a mono- and a bifunctional β-xylosidase (GH43) capable of hydrolysing p-nitrophenyl-α-L-arabinofuranoside and p-nitrophenyl-β-D-xylopyranoside from *P. purpurogenum.* Another β-xylosidase exhibiting optimum activity at 70 °C and pH 3.5 was purified from *A. pullulans* ATCC 20524. The enzyme showed 49% amino acid sequence similarity to GH 3 β-xylosidase reported from *T. emersonii* (Ohta et al. 2010). A GH 61 (60 kDa) enzyme, St Cel61a from *Sporotrichum thermophile*, was found efficient in boosting hydrolytic potential of a mixture of purified cellulases (EGII, CBHI, βG) by 20% (Dimarogona et al. 2012). Cellobiose dehydrogenase (CDH), which is actively involved in oxidative (CDH-LPMO) system for degradation of crystalline cellulose, has been purified from *T. hirsuta* (Nakagame et al. 2006) and *Termitomyces clypeatus* (Saha et al. 2008). There have been several reports for purification and characterization of laccase enzyme from *Pycnoporus sanguineus* (Ramírez-Cavazos et al. 2014), *Sinorhizobium meliloti* (Pawlik et al. 2016), *Bacillus licheniformis* (Koschorreck et al. 2008), *Fusarium solani* MAS2 (Wu et al. 2010), *T. versicolor* (Li et al. 2014) and *T. hirsuta* Bm-2 (Castillo et al. 2012).

It has been observed that purification of enzymes is a labourious and time-intensive process that requires a series of chromatographic steps; therefore, there is a need to devise methodologies for rapid purification of novel and catalytically active enzymes. In this context, Rai et al. (2016a) devised a rapid method by employing preparative SDS-PAGE in tandem microtitre plate-based enzyme assays to

purify and characterize an array of GHs including four functionally distinct isoforms of EG, two xylanase, one CBHI, Afase and β-xylo from *Acrophialophora* sp. P2.

16.7 Approaches for Developing Hyper-cellulolytic Strains

The recombinant strategies involving heterologous expression of the functional enzyme system (cellulases, hemicellulases and auxiliary enzymes) are being utilized extensively for efficient degradation of LCB. A novel endo-1,4-β-D-glucanase (EG) was recently cloned from *T. virens* ZY-01 and expressed heterologously in *E. coli* (Zeng et al. 2016). The cloned gene was 1069 bp long and showed 95.2% similarity with EG IV from *T. viride* AS 3.3711. A cellobiohydrolase B (*cbhB*) was cloned from *A. niger* ATCC 10574 and expressed into *E. coli* (Woon et al. 2015). The full-length cDNA of *cbhB* was cloned into cloning vector, pGEM-T Easy. After removing the signal peptide, cDNA of *cbhB* was cloned into expression vector pET-32b. The recombinant CbhB was expressed in *E. coli* Origami DE3 as an insoluble protein. A gene coding for β-xylosidase from *Geobacillus* sp. WSUCF1 was cloned and expressed in *E. cloni* 10 G chemically competent cells through pRham N-His SUMO Kan Vector (Bhalla et al. 2014b). The expressed protein was highly thermostable exhibiting $t_{1/2}$ of 9 days at 70 °C. A novel thermostable endoxylanase from *Geobacillus* sp. WSUCF1 was also cloned into pRham N-His SUMO Kan Vector and expressed in *E. cloni* 10 G cells (Bhalla et al. 2014a). The expressed enzyme showed $t_{1/2}$ of 60 h at 60 °C. The soluble flavin domain of *Phanerochaete chrysosporium* CDH expressed in *E. coli* exhibited higher dye-mediated activity as compared to the complete CDH (flavin domain and haem domain) prepared using *P. pastoris* as host (Ferri and Sode 2010).

Although recombinant protein expression is the most accepted technology for production of enzymes at the industrial level, the fungal lignocellulolytic enzyme titres are enhanced through strain improvement (Cherry and Fidantsef 2003; Gusakov 2011). Despite producing copious amounts of cellulases and hemicellulases, some wild-type fungal strains fall short of being considered ideal for commercial use. Therefore, these strains are continuously subjected to strain improvement programmes for improving their titre, catalytic and hydrolytic efficiencies. The cyclic mutagenesis involving physical and chemical mutagens followed by rational screening approaches are being employed to enhance the production of cellulases and other cell wall-degrading enzymes (Fang et al. 2009; Liu et al. 2013b; Ottenheim et al. 2015). UV radiation is the widely utilized physical treatment strategy, whereas chemical treatment uses mutagens like N-methyl-N'nitro-N-nitrosoguanidine (MNNG), acriflavin and ethanomethane sulphate (EMS). The studies have shown that these mutagens can be used alone or in combinations. There are several reports where combination of both physical and chemical treatments resulted in the overproduction of cellulolytic enzymes (Chadha et al. 2005; Adsul et al. 2007; Kaur et al. 2014). The developed mutants are picked on the basis of their phenotypic traits such as antimetabolite resistance and morphological and developmental differences (Chadha et al. 2005). The carbon catabolite

repression (CCR) is known to regulate the synthesis of cellulases where expression of various transcription factors, kinases and phosphatases play an important role in controlling cellular energy states (Ruijter and Visser 1997; Brown et al. 2013). Mutations in carbon repressor gene *Cre1* (Ilmen et al. 1996), β-glucosidase regulator gene *bglR* (Nitta et al. 2012) and glucosidase IIα subunit gene *gls* 2α (Geysens et al. 2005) have contributed to hyper-production of cellulolytic enzymes in the mutants developed from *T. reesei.* The mutated *cre1* and *bglR* genes reduced catabolite repression and mutations in *gls2α* gene resulted in changed pattern of N-glycans on secreted proteins. In order to develop strains with resistance to catabolite repression, 2-deoxy-D-glucose (2DG) is extensively used as a selection marker (Kaur et al. 2002; Dillon et al. 2006; Kaur et al. 2014). 2DG is a toxic analogue of glucose that provokes rapid growth arrest through inhibition of glycolytic pathway and glycosylation (Ralser et al. 2008). A study on *A. cellulolyticus* showed that *CreA* gene functions as a catabolite repressor protein and disruption of this gene enhances the disruption of cellulases and xylanases (Fujii et al. 2013). *CreB*, *CreC* and *CreD* genes in *A. nidulans* are also known for their active participation in CCR (Todd et al. 2000; Antonella et al. 2007). The mutation programmes are generally followed by several sequencing approaches for comparative analysis of parent and developed strains to figure out deletions or insertions in their nucleotide sequences. The expression profiling through SDS-PAGE also indicates the up- or downregulated proteins that can be identified through peptide mass fingerprinting (PMF) (Tian et al. 2009; Kaur et al. 2014).

The site-directed mutagenesis is another widely used approach for incorporating specific changes in a known DNA sequence through PCR-based techniques. Error-prone PCR (EP-PCR) introduces random mutation into a defined segment of DNA that is too long to be synthesized as a degenerate sequence chemically. EP-PCR followed by site-directed mutagenesis has been reported for generation of E289V variant from *B. amyloliquefaciens* DL-3 with 7.93-fold higher EG activity (Vu and Kim 2012). Wang et al. (2005) have used EP-PCR-based approach for increasing alkaline tolerance of EGIII in *T. reesei.*

Protoplast fusion is another powerful technique for introducing genetic modifications where two protoplasts are fused to form a hybrid cell. This technique has a great potential to develop industrially important microorganisms with minimal disturbances in their physiology (Prabavathy et al. 2006; Savitha et al. 2010). The isolation of protoplast involves digestion of cell wall by carbohydrase enzymes (Novozyme 234, chitinase, lysozyme, glucanase) in the presence of osmotic stabilizers (Gokhale et al. 1993; Kaur et al. 2013). A number of desirable genes from two or more complex parental genomes can then be recombined into a fusant through protoplast fusion at intraspecific, interspecific and intergeneric level. The fusion of protoplasts can be induced by mild electric stimulations (electrofusion) or chemicals such as polyethylene glycol (PEG), sodium nitrate and calcium ions (Pasha et al. 2007; Kordowska-Wiater et al. 2012). Interspecific protoplast fusion has been reported for successfully enhancing the cellulase production in *T. reesei* (Ogawa et al. 1989; Prabavathy et al. 2006) and developing heterokaryons between two cellulolytic strains *A. nidulans* and *A. tubingensis* (Kaur et al. 2013). Protoplast fusion

of *Rhizopus microsporus* led to a 1.46-fold increase in the production of fumaric acid from glycerol (2% w/v) as compared to the parental strains (Kordowska-Wiater et al. 2012). The protoplast fusion between the auxotrophic mutants of filamentous *R. cohnii* exhibited 3.5 times higher lipase activity than that of the original strain (Sawicka-Zukowska et al. 2004). This technique assists in inducing simultaneous genetic changes at different positions in the genome without any requirement of genome sequence information (Petri and Schmidt-Dannert 2004).

Recently, protoplast transformation has been identified as a potential platform for enhancing the cellulase production through RNA interference (RNAi) of *cre1* gene expression. Yang et al. (2015) studied the role of *cre1* in *M. thermophila* ATCC42464 using RNA interference. The *cre1*-silenced C88 strain exhibited 5.59-, 3.76-, 2.64- and 1.31-fold higher cellobiase, filter paper hydrolysing β-1,4-exoglucanase and β-1,4-endoglucanase activities, respectively, as compared to the parental strains. Another study targeting delivery of short-interference RNAs (SiRNAs) into the *Verticillium dahliae* protoplasts and their application in gene silencing was conducted by Rehman et al. (2016). In this study, protoplasts of Vd-GFP strain were transformed with different SiRNAs targeting the *GFP* gene. It was observed that SiRNA-gfp4 showed up to 100% gene silencing efficiency that lasted for at least 72 h.

Several morphological markers (colony morphology, spore size and shape) and genetic markers such as mycelial protein pattern, restriction digestion pattern and random amplified polymorphic DNA (RAPD) are used to analyse genetic recombinations. DNA polymorphism in pectinase overproducing fusants developed through interspecific protoplast fusion by *A. carbonarious* and *A. niger* was studied using RAPD (Kavitha and Umesh-Kumar 2000). 2D gel electrophoresis-based profiling of proteins showing presence or absence of protein bands in the parents and hybrids can be used effectively as a marker for determining genetic recombinations (Thangamani 2005; Savitha et al. 2010). Differential gene expression could be studied by employing serial analysis of gene expression (SAGE), subtractive suppressive hybridization (SSH) and rapid amplification of cDNA ends (RACE) (Velculescu et al. 1995; Chenchik et al. 1996; Oiatchenko et al. 1996).

16.8 Application of Cellulases in Biorefineries

The research on lignocellulolytic glycosyl hydrolases (GHs) is hotly pursued owing to their potential for bioconversion of LCB into 2G ethanol and other value-added products. These enzymes are produced by a diversity of microorganisms such as bacteria, yeast and fungi where filamentous fungi are the major producers. Several submerged fermentation (SmF) and solid-state fermentation (SSF) studies have been conducted for the production of cellulases and hemicellulases. The SSF is defined as fermentation with very little free water but substrate moist enough to support microbial growth (Pandey 2003), therefore mimicking the natural habitat of most of the filamentous fungi (Hansen et al. 2015). The current industrial demand of cellulases and hemicellulases is being met by culturing filamentous fungi using

SmF technology due to better control of the process parameters (Krishna 2005). The SmF technology has developed to a point where different parameters like temperature, pH, agitation and foam could be extensively controlled, depending upon specific requirements (Vaidyanathan et al. 1999). At industrial level, fed batch is the most preferred approach to achieve high protein yields. This is accomplished through feeding of monosaccharides (glucose and xylose), disaccharides (lactose) and polysaccharides (CMC) or a combination of these. Despite the fact that SmF is a highly developed technology, it is being claimed that enzyme titres are higher in SSF when comparing the same strain (Hansen et al. 2015). Therefore, there is a need to put attention on further developing and strengthening the SSF technology for commercial enzyme production due to its low capital cost, less energy utilization, ease of monitoring and low catabolite repression (Pandey et al. 2000; Aguilar et al. 2001).

Under SSF, *Thermoascus auranticus* produced 600 U/g of endoglucanase (de Silva et al. 2005) using corn cob as a carbon source. *P. echinulatum* 9A02S1 cultured on pretreated sugarcane bagasse showed 282 and 58.95 U/g of endoglucanase and β-glucosidase activity, respectively (Camassola and Dillon 2007). *A. niger* AT-3 exhibited 113.5 and 13.34 U/g endoglucanase and total cellulase (FPase) activity, respectively, when cultured on rice straw. *Malbranchea cinnamomea* produced 304, 187.8, 243.18 and 24000 U/g of EG, CBH, βG and xylanase, respectively, on wheat bran- and rice straw-solidified medium (Mahajan et al. 2016). *A. niger* NRRL 328 and *P. brasilianum* produced 950 and 709 U/g xylanase, respectively (Panagiotou et al. 2007; Montibeller et al. 2014).

Under SmF, *A. heteromorphus*, *Neurospora crassa* and *T. viridae* have been reported to produce 83, 19.7 and 33.8 U/ml of endoglucanase (Romero et al. 1999; Adsul et al. 2004; Singh et al. 2009a). *Penicillium* sp. Dal5 has been reported to produce 35.69, 2.86, 4.42 and 115 U/ml EG, CBH, βG and xylanases, respectively (Rai et al. 2016b). *P. oxalicum* JU-A10-T mutant, GS2-15 fusant, *Talaromyces thermophilus* and *P. janczewskii* produced 700, 226.3, 22 and 15.2 U/ml xylanases, respectively (Cheng et al. 2009; Terrasan et al. 2010; Guerfali et al. 2011; Yao et al. 2015).

The production of cell wall-degrading GHs is significantly influenced by a number of factors such as basal medium composition, carbon and nitrogen source, temperature, pH, additives and inoculum age. Rai et al. (2016b) demonstrated that basal medium played a crucial role in culturing *Penicillium* sp. Dal5 where replacing CWR medium with other media types significantly inhibited the expression of cellulases and xylanase. The choice of carbon source varies from one microorganism to another, e.g. rice straw supported high levels of cellulases and hemicellulases in *A. niger* KK2 (Kang et al. 2004), whereas pretreated sugarcane bagasse and wheat bran supported maximum levels of EG, FPase, βG and xylanse in *P. echinnulatum* (Camassola and Dillon 2007). The effect of different inorganic and organic nitrogen sources has been documented by many workers for the production of lignocellulolytic enzymes (Gautam et al. 2010; Lee et al. 2010). Operating conditions like temperature, pH and moisture are critical for microbial growth. Deviation in pH affects the ionization stage of nutrient molecules and, therefore, reduces their availability to microorganisms (Bibi et al. 2014). The decreased activity of enzymes at altered

pH may also result from destabilization of the produced enzymes. The moisture content also influences microbial growth and metabolism. Low moisture content reduces nutrient solubility causing lesser swelling of substrate, whereas increased moisture restricts the oxygen transfer to substrate by decreasing its porosity (Shah et al. 2017). The optimum temperature for expression of proteins is generally similar to the optimum temperature of microbial growth.

16.9 Enzymatic Hydrolysis

Bioconversion of LCB into 2G ethanol with enzymatic hydrolysis being a major bottleneck is the hot area of research over the past couple of decades. Several reviews addressing the technical issues associated with bioconversion at the commercial level have been published in the past decade (Hahn-Hägerdal et al. 2006; Lin and Tanaka 2006; Galbe and Zacchi 2007; Schmer et al. 2008; Margeot et al. 2009; Sukumaran et al. 2010). Companies like Iogen, Broin and Abengoa are setting up biorefineries for processing LCB into ethanol, and industries like Diversa, DuPont, Dyadic and Novozymes are focusing on the production of commercial cellulase/hemicellulase blends to support 2G ethanol (Chandel et al. 2010). DuPont has developed a range of enzyme mixtures Accellarase 1500 (Cellulase blend), Accellarase BG (β-glucosidase) and Accellarase XC (accessory xylanase/cellulase enzyme complex) for enhanced xylan (C5) and glucan (C6) conversion. Novozyme has also introduced a wide range of enzyme preparations such as Cellusoft AP and Cellusoft CR (textile industry), carezyme and care clean (detergent industry), Cellic CTec2, Cellic CTec3 and Cellic HTec3 (cellulase/hemicellulase blends). These high-performing commercial enzyme preparations are expensive being protected under patent regimes. Therefore, indigenous enzyme preparations with better catalytic and hydrolytic efficiencies at low concentrations are desired so as to reduce overall cost of the process. In this direction, many cellulases and hemicellulases derived from diverse microbial sources have been characterized for the efficient hydrolysis of differently pretreated substrates such as rice straw, wheat straw, sugarcane bagasse, corn cob, etc. The strain name CF-2612 developed through cyclic mutagenesis of *A. cellulolyticus* showed significantly higher yields of glucose (333.2 mg/g) from the hydrolysis of *Eucalyptus* wood chips. Such yields of glucose were attributed to higher βG/FPase ratio of the strain (Fang et al. 2009). The cellulases produced by *T. reesei* ZM4-F3 have been reported for degradation of 68.21% of the pretreated rice straw after 120 h hydrolysis (Zhang and Cai 2008). The cellulase mixture obtained from *Penicillium* sp. was found superior in hydrolysing α-cellulose derived from steam explosion of bagasse, pulverized cellulose (CP-123), microcrystalline cellulose and solka floc when compared to commercial cellulase mixture Accellarase (Genencore), at the same enzyme loading of 20 FPU/g under similar conditions (Singh et al. 2009b). A comparative study by Jørgensen et al. 2005showed superiority of cellulases derived from three *Penicillia*: *P. pinophilum* IBT 4186, *P. persicinum* IBT 13226 and *P. brasilianum* IBT 20888, over the commercial enzyme preparation Celluclast for hydrolysis of steam-pretreated

spruce at equal enzyme loading (25 FPU/g cellulose). The comparison of enzyme mixtures secreted by novel *Penicillium* and *T. reesei* strains with *T. reesei* reference strain revealed that *P. pulvillorum* TUBF-2220 is better for the hydrolysis of Avicel in comparison to the reference strain, whereas hydrolytic yields from pretreated spruce and wheat straw were quite similar (Marjamaa et al. 2013). The cellulase produced by *P. oxalicum* EU2106 showed glucan conversion of 93.6% after 96 h hydrolysis of sugarcane bagasse pulp at an enzyme loading of 20 FPU/g solid and substrate loading of 60 g/l (Huang et al. 2015). dos Reis et al. (2013) evaluated *P. echinulatum* enzymatic broth and commercial enzyme Celli CTec2 for the hydrolysis of pretreated sugarcane bagasse. The maximum glucose yields of 28% and 27% were observed using *P. echinulatum* enzyme and Cellic CTec 2, respectively.

16.10 Future Perspectives and Conclusions

Bioprospecting for novel cellulases from exotic niches using systems biology approaches in the future would play an important role in discovering unique cellulases and other auxiliary enzymes. The role of protein-protein interactions in enhancing the catalytic efficiency of cellulases is also foreseen. These approaches would further lower the cost of enzyme-based bioconversions.

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Secondary Metabolism in *Trichoderma*: Chemo- and Geno-Diversity

17

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Abstract

Trichoderma species are filamentous ascomycetous fungi that have wide biotechnological applications in industry as well as agriculture. Having nearly 300 species, this genus represents one of the most diverse groups of fungi. Secondary metabolites are useful natural products having widespread applications in agriculture and medicine. *Trichoderma* species are prolific producers of secondary metabolites (natural products) with proven role in disease suppression. Genes for biosynthesis of these metabolites are often present as gene clusters, and one such cluster may be responsible for synthesis of a range of metabolites and intermediates. Depending on the chemical nature, these metabolites could be grouped as non-ribosomal peptides, polyketides, terpenes, steroids, etc. Three species of *Trichoderma* (*T. virens*, *T. atroviride*, and *T. reesei*) are well studied from genomics point of view, and this article focuses mainly on these three species. We discuss here the level of diversity with respect to secondary metabolite biosynthesis machinery at the genus, species, and strain level with genetic evidence where available. The article highlights the untapped potential of *Trichoderma* spp. as a source of a variety of secondary metabolites with potential applications in agriculture and medicine.

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Secondary metabolism · *Trichoderma* · Viridin · Gliotoxin · Gliovirin · Peptaibols · Genomics

17.1 Introduction

Trichoderma spp. are among the most diverse group of fungi with nearly 300 defined species (Robbertse et al. 2017). These fungi are widely used in industry as source of enzymes and in agriculture as biofungicides and plant growth promoters (Mukherjee et al. 2013a, b). Several formulation products for agricultural usage are available worldwide, and in India alone, a few hundred products are there in the market (Singh et al. 2017). *Trichoderma* species are strong mycoparasites and can kill other fungi in contact (Druzhinina et al. 2011). These fungi are prolific producers of secondary metabolites/natural products which can be broadly classified as high molecular weight (peptaibols) and low molecular weight (non-ribosomal peptides, polyketides, terpenes, steroids, etc.). There are more than 1000 peptaibols reported from *Trichoderma*, and similarly, more than 1000 small molecular weight compounds are known to be produced by this single genus (Zeilinger et al. 2016). Secondary metabolites play an important role in *Trichoderma*-fungus and *Trichoderma*-plant interactions (Mukherjee et al. 2012a). These compounds may be used to weaken the prey fungus before mycoparasitic attack generally mediated by hydrolytic enzymes like chitinases, beta-glucanases, and proteases. Some of these compounds exhibit strong antimicrobial properties (antibiosis), while others may be involved in inducing resistance response against plant pathogens (Viterbo et al. 2007; Mukherjee et al. 2012b). *Trichoderma* spp. also produce phytohormones that promote plant growth (Contreras-Cornejo et al. 2009). In short, secondary metabolites are important components of the package of benefits that *Trichoderma* spp. provide to plants. In this article, we discuss about the diversity of secondary metabolites that these fungi produce and, where information is available, provide genetic evidence for the origin of such vast chemo-diversity in these beneficial filamentous fungi. We focus on three species (*T. atroviride*, *T. virens*, and *T. reesei*) as these have been well characterized subsequent to the publication of the whole genome sequences.

17.2 Secondary Metabolites of *Trichoderma* spp. and Their Biosynthesis

Trichoderma species produce a plethora of secondary metabolites belonging to various classes like non-ribosomal peptides, polyketides, terpenes, etc. Table 17.1 presents some examples of secondary metabolites produced by the three well-characterized species of *Trichoderma*.

Table 17.1 Some examples of secondary metabolites produced by *T. reesei*, *T. atroviride*, and *T. virens*

Chemical category	Compound	Species	Biological role
Non-ribosomal peptides			
Peptaibols	Trichovirin II	*T. virens*	Induces resistance in cucumber plants against a bacterial pathogen
Peptaibols	Trichorzianins Atroviridins A–C	*T. atroviride*	Atroviridins A–C are associated with conidiation
Peptaibols	Paracelsin, hypojecorins A and B	*T. reesei*	Paracelsin is reported to be hemolytic
Siderophore (intracellular)	Ferricrocin	*T. atroviride*, *T. virens*, *T. reesei*	Intracellular storage of iron, involved in gliotoxin biosynthesis and ISR
Siderophores (extracellular)	Fusarinines A–B Dimerum acid Fusigen Coprogen	*T. virens*	Iron acquisition, competition
Diketopiperazine/NRP	Gliotoxin	*T. virens*	Antiviral, antibacterial, fungistatic activity, anti-cancer and immuno-suppressive properties
Diketopiperazine	Gliovirin	*T. virens*	Antimicrobial compound against oomycetes and *Staphylococcus aureus*, antitumor
Dipeptide	Trichodermamide A, B	*T. virens*	Cytotoxicity
Polyketides			
Polyketides	Trichodermatides B–D	*T. reesei*	Cytotoxicity
Polyketide	Conidial pigment	*T. reesei*	Conidial pigmentation, stress tolerance
Polyketides	Trichorenins A–C	*T. virens*	Algicidal
Polyketide	Sorbicillin	*T. reesei*	Antiviral, anti-inflammatory, and antimicrobial activities
Terpenes and steroidal compounds			
Sesquiterpene	Heptelidic acid (koningic acid)	*T. virens*	Potential activity against the human malaria parasite *Plasmodium falciparum*, antimicrobial, anticancer
Sesquiterpene	*β*-Farnesene	*T. atroviride*	Acts as an alarm pheromone in aphids
Sesquiterpene	*β*-Caryophyllene	*T. virens*	Attracts nematodes that prey on insect larvae
Monoterpene	*β*-Myrcene	*T. virens*	Regulates the expression of genes related to abiotic and biotic stresses

(continued)

Table 17.1 (continued)

Chemical category	Compound	Species	Biological role
Monoterpenes	*Cis*- and *trans*-*β*-Ocimene	*T. virens*	Induces expression of JA defense response-related genes in *A. thaliana*
Steroidal compound	Viridin	*T. virens*	Antifungal metabolite that alters the spore germination of *Botrytis allii*, *Colletotrichum lini*, and *Fusarium caeruleum*
Other compounds			
Indolic compound	Indole-3-acetic acid (IAA)	*T. atroviride*, *T. virens*	Plant growth promotion
Indolic compound	Indole-3-acetaldehyde	*T. atroviride*, *T. virens*	Plant growth promotion
Indolic compound	Indole-3-carboxaldehyde	*T. atroviride*, *T. virens*	Induces adventitious root formation in *A. thaliana*
Carotanes	Trichocaranes A–D	*T. virens*	Inhibits the growth of etiolated wheat coleoptiles
Pyrone	6-Pentyl-2*H*-pyran-2-one	*T. atroviride*	Antifungal, antinematode, and plant growth-promoting activities in tomato and *A. thaliana*
Ketone	3-Octanone	*T. atroviride*	Induces conidiation
Alcohol	1-Octen-3-ol	*T. atroviride*	Induces conidiation and defense responses in plants through JA

Source: Reino et al. (2008), Ruiz et al. (2013) and Contreras-Cornejo et al. (2016)

17.2.1 Non-ribosomal Peptides (NRPs)

The non-ribosomal peptides contain both proteinogenic and non-proteinogenic amino acids and may exist in linear or cyclic form. They are synthesized by multi-modular non-ribosomal peptide synthetases. Each module includes adenylation, peptidyl carrier, and condensation domains. The important NRPs produced by *Trichoderma* spp. are peptaibols, epipolythiodioxopiperazines (ETPs), and siderophores.

17.2.1.1 Peptaibols

Peptaibols are short peptides containing α-aminoisobutyric acid (Aib) and a C-terminal alcohol. These are the most prominent NRPs produced by *Trichoderma* species. Peptaibols are reported to have antimicrobial property and cytotoxic activity, and these can induce systemic resistance in plants. The antibiotic property of peptaibols is majorly due to amphipathic nature of peptaibols which allow concentration-dependent membrane permeabilizing activity (Bortolus et al. 2013). Peptaibols are synthesized by peptaibol synthetases (NRPSs) consisting of different modules. Seven-, 14-, and 18–20-module peptaibol synthetases are present in *Trichoderma* genomes (Mukherjee et al. 2012b). The first peptaibol synthetase enzyme (Tex1) has been reported in *Trichoderma virens* (Wiest et al. 2002). Tex1 is an 18-module peptaibol synthetase and produces 18-residue trichovirin II type peptaibols. *T. virens* also possess 14-module peptaibol synthetase enzyme which

produces two classes of peptaibol (the 11- and 14-residue peptaibols) [Mukherjee et al. 2011]. *T. atroviride* is reported to produce 19-residue atroviridins which are produced by a 19-module peptaibol synthetase. Like *T. virens*, *T. atroviride* and *T. reesei* also produce 11- and 14-residue peptaibols catalyzed by a 14-module peptaibol synthetase (Degenkolb et al. 2012).

17.2.1.2 Epipolythiodioxopiperazines (ETPs)

Epipolythiodioxopiperazines (ETPs) are secondary metabolites with a characteristic cyclic peptide-derived diketopiperazine ring. Gliotoxin and gliovirin are members of the ETP class of peptides. Gliotoxin is produced by "Q" strains of *T. virens*, whereas gliovirin is produced by "P" strains of *T. virens* (Howell et al. 1993). Gliotoxin has antimicrobial activity and fungistatic property. In *Aspergillus fumigatus* which is a human pathogen, the gliotoxin acts as a virulence factor (Scharf et al. 2016). GliP is the NRPS dioxopiperazine synthetase enzyme involved in the biosynthesis of gliotoxin. The gliotoxin biosynthetic gene cluster in *A. fumigatus* consists of 12 genes inclusive of GliP gene, while in *T. virens*, the gliotoxin gene cluster consists of only eight genes (Fig. 17.1). In *T. virens*, the deletion of part of the *gliP* gene confirmed the role of it in gliotoxin production (Vargas et al. 2014). The gliotoxin biosynthetic gene cluster with only six genes was also identified in *T. reesei*, albeit this species is not reported to produce gliotoxin (Mukherjee et al. 2012a). The *T. virens* genome also contains a putative SirP gene cluster which is associated with the production of phytotoxin sirodesmin PL in the phytopathogen *Leptosphaeria maculans*. However, the product of SirP gene cluster is not known yet in *Trichoderma*. We have recently discovered the *glv* gene cluster responsible for gliovirin biosynthesis in "P" strains of *T. virens* (Sherkhane et al. 2017).

17.2.1.3 Siderophores

Siderophores are secondary metabolites that can bind, transport, and store iron. Siderophore-mediated iron acquisition is important for microbial competition, biocontrol, and in interactions with plants and other microbes. There are two types of siderophores, intracellular and extracellular. Ferricrocin is an intracellular siderophore and is reported to protect cells from oxidation-induced stress. The product of

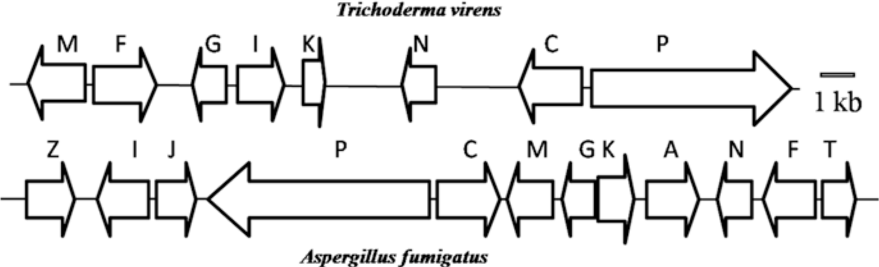

Fig. 17.1 The gliotoxin biosynthesis cluster of *Trichoderma virens* and *Aspergillus fumigatus* – P, non-ribosomal peptide synthetase (NRPS); G, glutathione-S-transferase; J, dipeptidase; N, N-methyl transferase; F and C, cytochrome P450; M, O-methyl transferase; I, C-S-bond lyase; K, gamma-glutamate cyclotransferase. (Adapted from Zeilinger et al. 2016)

NPS6 is an extracellular siderophore that acts as virulence factor in *Cochliobolus heterostrophus* as well as protects fungus from oxidative stress. In *Trichoderma* spp., three different NRPSs involved in siderophore biosynthesis have been identified. Intracellular ferricrocin-associated gene cluster has been found in all the three *Trichoderma* species, but the role for the gene for ferricrocin biosynthesis has been established only in *T. virens* (Mukherjee et al. 2018). NPS6 and SidD are the NRPSs reported to be involved in extracellular siderophore biosynthesis. A gene cluster with NPS6 as a core enzyme is found in all the three *Trichoderma* species, but the function is confirmed only in *T. virens* with gene deletion experiment (Mukherjee et al. 2013a). Another gene cluster with SidD as a core enzyme is found only in *T. virens* and *T. reesei* (Mukherjee et al. 2012b)

17.2.2 Polyketides

Polyketides are secondary metabolites, many of which are having antimicrobial and anti-cancer property. Some polyketides are important for competition for substrate and for interaction with other organisms. Polyketides are synthesized by the polyketide synthases, which are complex enzymes with ketoacyl synthase, an acyl transferase, and a phosphopantetheine attachment site domain. Few studies have been published on the biosynthesis and functional role of polyketides in *Trichoderma* species, although the genomes of *Trichoderma* species are rich in PKS-encoding genes. Orthologues of PKS genes associated with the conidial pigment biosynthesis cluster have been identified in all the three *Trichoderma* species (Baker et al. 2012). Additional 20 putative PKS gene clusters have also been reported in these three species (Bansal and Mukherjee 2016). The role of PKS genes in green pigmentation of conidia, teleomorphic structure, conidial cell wall stability, and antagonistic abilities has been confirmed in *T. reesei* by deletion of *pks4* gene which is an orthologue of pigment forming PKS in *Fusarium* spp.

17.2.3 PK/NRPs

Several PKS-NRPS hybrid enzymes are present in *Trichoderma* genomes. Functional study provided evidence for the role of one of the PKS-NRPS hybrid enzymes (Tex13) in inducing the defense-related *pal* gene in maize seedlings. The metabolite produced by Tex13 cluster is still not known (Mukherjee et al. 2012b).

17.2.4 Terpenoids

Terpenoids represent a diverse class of secondary metabolites produced by almost all the organisms including fungi. They are composed of five-carbon isoprene units (C_5H_8) producing hemiterpenes, monoterpenes, sesquiterpenes, diterpenes, sesterterpenes, triterpenes, tetraterpenes, or polyterpenes. Terpenoid biosynthetic gene clusters have terpene cyclase as the core enzyme. Only a few terpene cyclases may

be responsible for production of diverse form of terpenoids. *Trichoderma* species are reported to produce all forms of terpenoids like volatile terpenoids, diterpenes, sesquiterpenes, and triterpenes (Zeilinger et al. 2016).

In most organisms, the mevalonate pathway is responsible for the formation of isoprene units (Zeilinger et al. 2016). Hydroxy-methylglutaryl-CoA reductase (HMGR) encoded by *hmgR* gene is the first enzyme in the mevalonate pathway involved in conversion of hydroxy-methylglutaryl-CoA into mevalonate. Deletion of *hmgR* gene in *Trichoderma harzianum* showed reduction in antifungal activity against *Rhizoctonia solani* and *Fusarium oxysporum* and decrease in ergosterol levels. Ergosterol encoding gene (*erg1*) silencing also showed decrease in the ergosterol level in *T. harzianum*, whereas overexpression of erg1 gene increases the antifungal activity of *T. harzianum* (Cardoza et al. 2006, 2014). The genome analysis of *T. reesei*, *T. atroviride*, and *T. virens* revealed that *T. virens* (11) has the highest number of terpene cyclases followed by *T. atroviride* (7) and *T. reesei* (6), but the terpene cyclases associated with the biosynthetic gene cluster are six in *T. virens*, three in *T. atroviride*, and two in *T. reesei* (Bansal and Mukherjee 2016). The first terpenoid biosynthetic gene cluster was identified in *T. virens* using suppression subtractive hybridization technique (Mukherjee et al. 2006). The cluster was initially predicted to be associated with viridin production but later found to be responsible for biosynthesis of volatile sesquiterpenes (Crutcher et al. 2013). Deletion of terpene cyclase present in the cluster abolished the production of all the volatile sesquiterpene compounds. This cluster named as *vir* cluster was found to be present in *T. virens* and in few *Aspergillus* species but not in other species of *Trichoderma*. The reason for existence of the *vir* cluster in distantly related *Trichoderma* and *Aspergillus* species could be explained by horizontal gene transfer. Another terpene cyclase Tri5 is responsible for the production of a phytotoxic agent, trichodermin, in *Trichoderma brevicompactum*. Overexpression of Tri5 enhanced the production of trichodermin in *T. brevicompactum* (Tijerino et al. 2011a, b).

17.2.5 Steroids

Viridin is a triterpene steroidal metabolite produced by both “P” and “Q” strains of *T. virens*. It has antifungal and anticancer properties. The reduced form of viridin is known as viridiol. Viridiol has herbicidal properties (Jones and Hancock 1987). Both viridin and viridiol are produced abundantly by *T. virens*, and the *vdn* cluster for viridin biosynthesis has recently been discovered (Fig. 17.2). Interestingly, an orthologous gene cluster is also present in the bat white nose fungus *Pseudogymnoascus destructans* (Bansal et al. 2018).

17.2.6 6-Pentyl Pyrone (6-PP)

6-PP belongs to the volatile class of secondary metabolites. The characteristic “coconut aroma” produced by some *Trichoderma* species is due to 6-PP metabolite production. 6-PP has antifungal and plant growth-promoting property (Vinale et al.

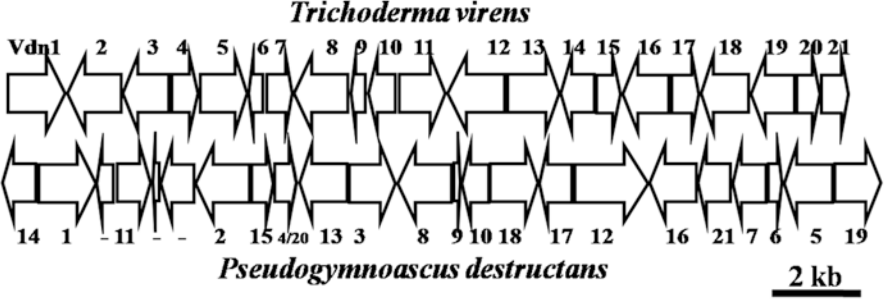

Fig. 17.2 The viridin-biosynthesis gene cluster of *Trichoderma virens* and its orthologue in *Pseudogymnoascus destructans.* (Adapted from Bansal et al. 2018). For details, please refer to Bansal et al. (2018)

Table 17.2 Secondary metabolism related core genes in the genomes of *T. reesei*, *T. atroviride*, and *T. virens*

Core genes	*T. reesei*	*T. atroviride*	*T. virens*
NRPS (with at least one complete module)	8	9	22
PKS	11	15	18
PKS/NRPS	2	1	4
Terpene cyclase	6	7	11

Source: Zeilinger et al. (2016)

2008). There is not much information available on the biosynthetic pathway for the 6-PP production, but a lipoxygenase enzyme has been predicted to be involved in the 6-PP biosynthesis as it is present exclusively in *T. atroviride*, but not in other *Trichoderma* species which are devoid of 6-PP production like *T. virens* and *T. reesei* (Kubicek et al. 2011).

17.3 Secondary Metabolism Genes and Gene Clusters: Diversity at the Genus Level

In order to have a comparative assessment of why *T. atroviride* and *T. virens* are strong mycoparasites compared to *T. reesei*, a comparative genomics analysis was performed, and it was found that *Trichoderma atroviride* and *Trichoderma virens*, the two aggressive mycoparasites, harbor more genes for hydrolytic enzymes like chitinases and glucanases (Kubicek et al. 2011). Interestingly, however, these two genomes also are richer in secondary metabolite biosynthesis genes (Table 17.2). Many of the secondary metabolite biosynthetic genes form gene clusters inclusive of core enzymes such as non-ribosomal peptide synthetases (NRPSs), polyketide synthases (PKSs), terpene synthases/cyclases, and others. These gene clusters harbor other genes as well like cytochrome P450s, oxidoreductases, methyl transferases, genes for transporters, and transcription factors (Bansal and Mukherjee 2016).

17.4 Intra-species Diversity in Secondary Metabolites

Trichoderma virens produces a plethora of secondary metabolites, some of which may be strain specific (Howell et al. 1993). Gliotoxin and gliovirin are the best examples showing intra-species diversity of secondary metabolites in *T. virens*. As discussed above, there are two strains of *T. virens*, P and Q. "Q" strains of *T. virens* produce gliotoxin, while "P" strains produce gliovirin (Fig. 17.3). Genome of *T. virens* "Q" strain Gv29-8 had already been sequenced (Kubicek et al. 2011), and by mining this genome, the gliotoxin biosynthesis gene cluster could easily be identified as information on gliotoxin gene cluster in *Aspergillus fumigatus* was already known (Mukherjee et al. 2012a). However, the gliovirin gene cluster remained elusive until the whole genome of a "P" strain (IMI 304061, a strain isolated from India) was sequenced by us. By comparative genome analysis, we were able to identify the whole cluster responsible for biosynthesis of gliovirin; its biosynthetic role was confirmed by gene knockout and LC-MS/MS analysis (Sherkhane et al. 2017). Gliotoxin gene cluster is absent in "P" strain. Interestingly, an orthologue of the gliovirin cluster is present in a distantly related fungus *Aspergillus udagawae* (Fig. 17.4).

17.5 One Gene Cluster: Many Metabolites

The diversity in secondary metabolites is also brought about by single gene cluster. In *T. virens*, the *vir* cluster has been reported to produce 22 volatile secondary metabolites. These include both monoterpenes and sesquiterpenes. The volatile compounds produced by the *vir* cluster have important roles. For example, beta-caryophyllene has anti-inflammatory and antimicrobial property, and germacrene D has antioxidant and antibacterial property. Interestingly, a glyceraldehyde 3 phosphate dehydrogenase (GAPDH) was found to be present in the *vir* cluster in both *Trichoderma* and *Aspergillus* species (Mukherjee et al. 2006). This GAPDH is isomer of the GAPDH involved in glycolysis in the genome of *Trichoderma* and *Aspergillus* species. Deletion of GAPDH in the *vir* cluster confirms its role in volatile sesquiterpene compound biosynthesis (Fig. 17.5) [Pachauri et al. 2018].

17.6 One Gene: Many Metabolites

Deletion of *tex2* encoding a 14-module non-ribosomal peptide synthetase (NRPS) resulted in abolition of two types of peptaibols, 14-residue peptaibols and 11-residue peptaibols. A total of 88 peptaibols were reported to be produced by Tex2 (53 14-residue peptaibols and 35 11-residue peptaibols). Module skipping and degeneracy gave rise to such diversity (Fig. 17.6).

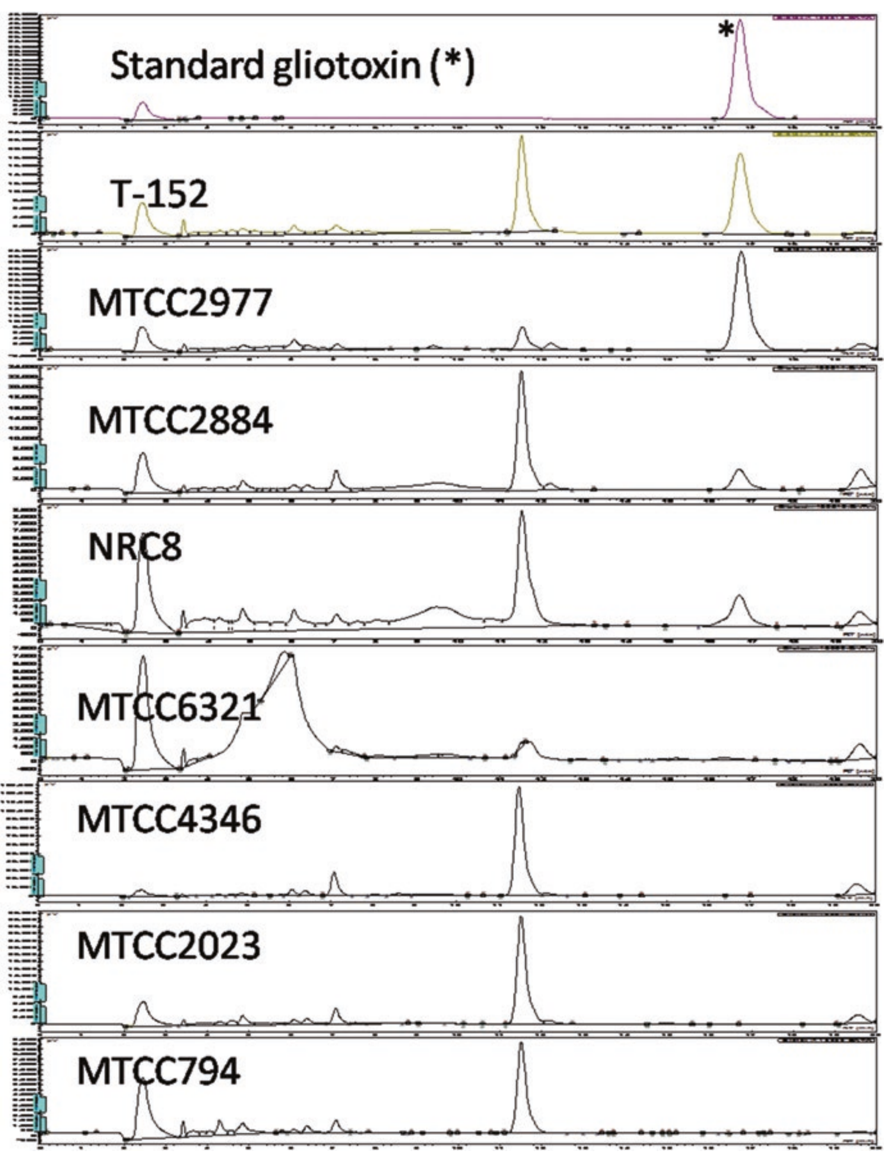

Fig. 17.3 HPLC analysis of filtrates of *T. virens* strains. Note the presence of gliotoxin in "Q" strains (T-152, MTCC 2977, MTCC 2884, NRC 8) and its absence in "P" strains

17.7 Regulation of Secondary Metabolism

Secondary metabolite diversity may also be regulated by environmental conditions including biotic and abiotic stresses. Though several gene clusters are present in a genome, only a few are expressed under standard laboratory cultivation conditions.

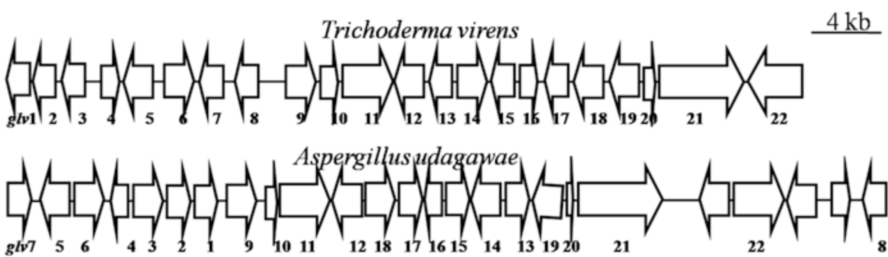

Fig. 17.4 The gliovirin gene cluster of *T. virens* and its orthologous cluster in *A. udagawae*. (Adapted from Sherkhane et al. 2017). Please refer to Sherkhane et al. (2017) for details

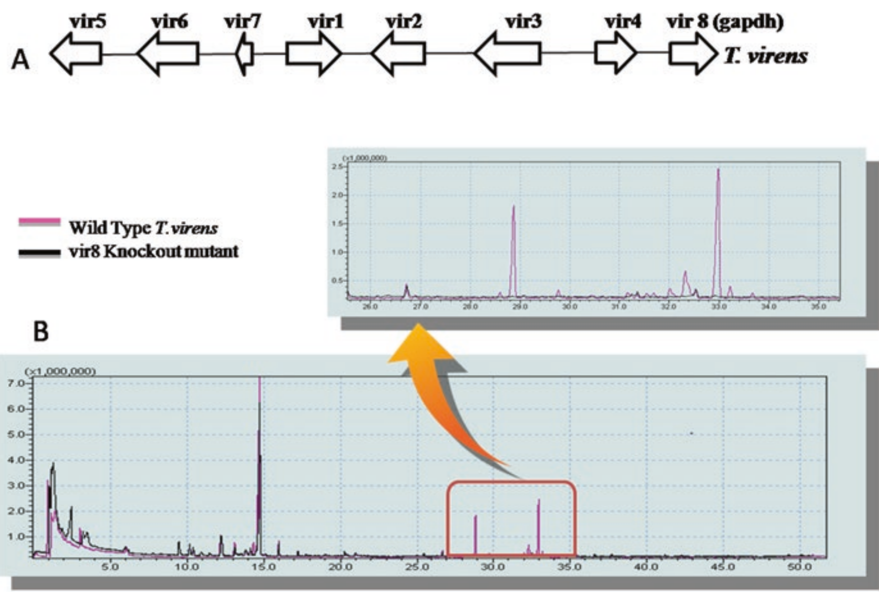

Fig. 17.5 (**a**) The *vir* cluster responsible for production of volatile sesquiterpene compounds in *Trichoderma virens*. (**b**) GC-MS profile of wild-type *T. virens* and *vir8* knockout mutant. No volatile sesquiterpene compounds are produced by *vir8* knockout mutant. (Adapted from Pachauri et al. 2018)

Inducing the expression of such gene clusters by using biological or genetic tools gives rise to chemo-diversity (Brakhage and Schroeckh 2011). Many secondary metabolite biosynthetic gene clusters possess putative transcription factors. Thctf1 is a transcription factor reported to be associated with the production of 6-PP in *T. harzianum*. Deletion of Thctf1 in *T. harzianum* resulted in decrease in the expression of two secondary metabolites derived from 6-PP and altered antimicrobial activity (Rubio et al. 2009). The secondary metabolites produced by *Trichoderma* species are reported to be influenced by other microorganisms, pH signaling, and the velvet-complex proteins. The interaction of *T. atroviride*, *T. virens*, and *T. reesei* with *Rhizoctonia solani* was studied using transcriptome analysis, and this analysis

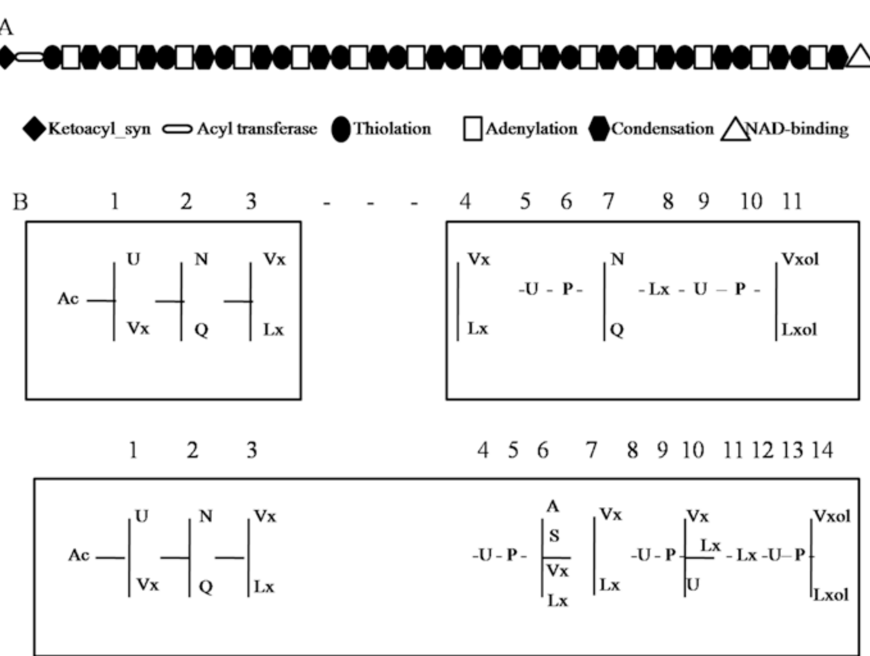

Fig. 17.6 (**a**) Modular organization of peptaibol synthetase Tex2. (**b**) sequence alignments of 11- and 14-residue peptaibols produced by *T. virens*. Sequences are given in standard single-letter code (Ac, acetyl-, U, Aib, Vx, Val/Iva, Lx, Leu/Ile, and ol represents the C-terminal amino-alcohol). The proposed module order is represented by numbers above each box. (Adapted from Mukherjee et al. 2011)

highlighted important genes influenced by the presence of *R. solani*. Interaction of *T. atroviride* and *T. reesei* with *R. solani* showed upregulation of two PKSs (Atanasova et al. 2013; Kubicek et al. 2011). In *T. atroviride*, the presence of *R. solani* also upregulates a 6-PP biosynthesis-related lipoxygenase gene (Kubicek*et al* 2011). In *T. virens*, the presence of *R. solani* upregulates expression of all the genes associated with the gliotoxin biosynthetic gene cluster (Atanasova et al. 2013). In another report, the presence of mycotoxin fusaric acid (FA) produced by *Fusarium* reduced the production of 6-PP and increased the 1-octen-3-ol biosynthesis. But in certain *Trichoderma* strains, the presence of fusaric acid does not alter the volatile profile; instead, 6-PP production inhibits FA production (Stoppacher et al. 2010). Change in the pH of the environment causes induction of a pH regulator PacC which further regulates expression of many genes in the fungus. For example, the deletion of a PacC orthologue in *T. virens* alters the expression of genes associated with secondary metabolite biosynthesis and iron transport, and the mutant also had decreased biocontrol activity (Mukherjee et al. 2012a). The velvet complex is best studied in *Aspergillus nidulans* and includes a methyltransferase LaeA and the two velvet proteins VeA and VelB. The velvet complex is responsible for coupling light response to the regulation of sexual development and secondary metabolite

biosynthesis. Vel1 is an orthologue of veA in *T. virens*, and deletion of vel1 gene in *T. virens* ceased the production of gliotoxin and downregulated many secondary metabolism-related genes (Mukherjee and Kenerley 2010). The *T. reesei* LaeA orthologue Lae1 is required for the expression of lignocellulose-degrading enzymes, and this enzyme is also found to be regulated epigenetically (Karimi-Aghcheh et al. 2013b). Lae1 gene deletion in *T. atroviride* resulted in the reduced expression of PKS-encoding genes and 6-PP-related lipoxygenase gene (Karimi-Aghcheh et al. 2013a). The mutant also displayed decreased mycoparasitic activity and reduction in the production of antifungal water-soluble metabolites and VOCs. The adenylyl cyclase-inhibiting Gα subunit is encoded by *tga1* gene, and the adenylyl cyclase-stimulating Gα subunit is encoded by *tga3* gene in *T. atroviride*. Deletion of *tga1* gene decreased the production of 6-PP, but the peptaibol production increased (Reithner et al. 2005), whereas deletion of *tga3* gene completely abolished the production of peptaibols, and its production was found to be regulated by two blue light regulators BLR1 and BLR2 in *T. atroviride* (Komon-Zelazowska et al. 2007). In *T. virens*, the role of *tac1* gene encoding adenylate cyclase has been identified (Mukherjee et al. 2007). MAPK-dependent signaling pathway was also shown to be involved in secondary metabolite biosynthesis and regulation. MAPK-encoding gene *tmk1* deletion in *T. atroviride* increased the production of peptaibols and 6-PP and also increased the antifungal activity (Reithner et al. 2007). But there was no change in secondary metabolites biosynthesis in *T. virens* in *tmk1* deletion mutants (Mendoza-Mendoza et al. 2003).

17.8 Conclusions

Trichoderma spp. are genetically diverse group of fungi which produce a plethora of secondary metabolites with known and unknown functions and applications. Tremendous amount of variability in secondary metabolite biosynthesis exists even within the same species. The genomes of these fungi are rich in secondary metabolism-related gene clusters, many of which are silent under standard laboratory culture conditions. With new genetic tools available, it is possible to induce the expression of such "silent" clusters which will add to the metabolic diversity of these fungi. Since many secondary metabolites are bioactive, it's possible to discover novel molecules from these "biocontrol fungi" that might actually find direct applications in agriculture and medical science.

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18 Diversity of Chitinase-Producing Bacteria and Their Possible Role in Plant Pest Control

Sandipan Banerjee and Narayan Chandra Mandal

Abstract

In nature, chitin is the second most plentiful and renewable polysaccharide and is present among versatile group of organisms from fungi and nematodes to arthropods and crustaceans. Enzymatic degradation is the preferable environmentally safe mode of bioprocessing of this inert biopolymer. Chitin-scavenging enzyme-producing sources are covering the living groups from prokaryotes to plants, viruses, vertebrates, and even human. Current-day biotechnologies have raised the development of bioprocesses by using microbes especially bacteria. Bacteria that produce chitinases are with varieties of habitats ranging from Antarctic soil to hot spring, crustacean waste site, animal gut, and endophytic ecosystems. Chitin metabolism is a necessary life-supporting goings-on in agronomic plant pests like fungi, insects, and parasitic nematodes which are negatively proportionate to the agricultural production systems. Placement of such potent chitinolytic bacteria for plant fortification against attacking pests is a well-practiced, biotechnologically equipped biocontrol strategy. By-products of chitin by enzymatic hydrolysis, like oligomers or monomers, have several applications in persuading the plant defense systems. Carrying the host-defensive activity to biocontrol potentiality against plant pests, bacteria with chitinolytic property also behaved as a plant growth-promoting biofertilizing employee in modern-day sustainable agricultural practices. In this context, the distribution of chitinase-producing bacteria according to their diversity of habitats is studied, and the less explored habitats can be an arsenal for biocontrolling agents against plant pests.

Keywords

Chitinase · Bacteria · Diversity · Biocontrol · Plant pest

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18.1 Introduction

Chitin is the second most abundant biodegradable carbon substrate after cellulose, which exists naturally in the biosphere as a structural polysaccharide of β-(1,4)-linked *N*-acetyl-D-glucosamine (GlcNAc). In nature, chitin is available in two crystalline formats, α and β. In the case of α-chitin, it is the most copious crystalline form, and the linear chains of GlcNAc unit are assembled in an antiparallel fashion, commonly exemplified by the shrimps and crabs, fungi, and cysts of *Entamoeba*. On the other hand, β-chitin is made up of parallel chains of GlcNAc units and found in squid pens (Yan and Fong 2015; Jang et al. 2004). Overall, chitin is extensively distributed in nature, mainly as an organizational polysaccharide in fungal cell walls (predominantly in *Ascomycota*, *Basidiomycota*, and *Chytridiomycota*), exoskeletons of arthropods, external shells of crustaceans, egg shell, and gut lining of parasitic nematodes (Brzezinska et al. 2014; Lenardon et al. 2010). The applicable fields of chitin are biotechnologically noteworthy, from chemical, biochemical, food, and pharmaceutical (antimicrobial, anticholesterol, antitumor, drug delivery, dietary fiber, and wound healing) industries (Patil et al. 2000; Gooday 1999; Muzzarelli et al. 1999; Dixon 1995) to wastewater treatment and management (Flach et al. 1992).

The insolubility of chitin and its inertness to chemical agents have amplified the exploration for substitute disposal methods such as biological processing. One such preferable practice is enzymatic treatment because of its uniformity toward the reaction and the products. Oligomers or monomers, by-products of chitin, have several applications in eclectic arenas (Patil et al. 2000). For such bio-based handling, chitinase comes first, and it acts to hydrolyze the β-1,4-glycosidic bonds between the *N*-acetyl-D-glucosamine residues that encompass a chitin chain (Henrissat 1999). Chitinases are classified into two types, exochitinases and endochitinases, based on their site and the nature of their hydrolyzed bonds (Henrissat 1999; Henrissat and Bairoch 1996). Endochitinases cleave chitin chains in random locations, generating low molecular weight oligomers, such as chitotriose, chitotetraose, and diacetylchitobiose. The exochitinases have been alienated into two subcategories: chitobiosidases which gradually release diacetylchitobiose from the non-reducing end of the chitin and β-*N*-acetylglucosaminidases, cleaving the oligomers of chitin (products of endochitinase), thereby producing monomers of glucosamine (Hamid et al. 2013).

Chitinases so far sequenced are also classified into glycoside hydrolase families (families 18, 19, and 20), constructed on the basis of amino acid sequence resemblance of their catalytic domains. The chitinases with different family backgrounds have dissimilar amino acid sequence and completely unlike three-dimensional (3D) structures (Perrakis et al. 1994; Henrissat 1991) and molecular mechanisms. Therefore, they are likely to have evolved from diverse lineages. The family 18 chitinases hydrolyze glycosidic bonds with the retention of anomeric configuration at C1 atom (Kramer and Koga 1986). The catalytic domains of these chitinases have a fold of barrel with a catalytic groove as demonstrated by 3D structural analysis of hevamine (Kramer and Muthukrishnan 1997). These chitinases catalyze the hydrolysis of Glc-N-Ac-Glc-N-Ac and Glc-N-Ac-Glc-N- linkages. These chitinases are inhibited by allosamidine, an isomer of *N*-acetyl glucosamine. On the other hand,

the family 19 chitinases hydrolyze glycosidic bond with an inversion of anomeric configuration at C1 atom (Stinizi et al. 1993; Broglie et al. 1991). The catalytic domain of these chitinases has a fold of high helical content and structural similarity, including conserved core of the enzyme (Grison et al. 1996). They catalyze the hydrolysis of Glu-N-Ac and Gluc-N-Ac linkages only. The activity of these chitinases is insensitive to allosamidine. They catalyze the hydrolysis of chitin similar to acid-base mechanism (Grison et al. 1996; Desouza and Murray 1995). The conserved region of the catalytic domain of this family of chitinases resembles crystal structure of lysozyme (Terwisscha et al. 1996). Family 18 (subfamilies A, B, and C) includes chitinases derived mostly from fungi but also from bacteria, viruses, animals, insects, and plants. Family 19 comprises chitinases derived from plants (classes I, II, and IV), and several are derived from bacteria, e.g., *Streptomyces griseus*. Family 20 includes *N*-acetylglucosaminidase from *Vibrio harveyi* and *N*-acetylhexosaminidase from *Dictyostelium discoideum* and human (Brzezinska et al. 2014; Dahiya et al. 2006; Duo-Chuan 2006; Patil et al. 2000; Henrissat 1999). Largely, chitinases produced by a versatile group of living systems range from microbes like bacteria, fungi, and virus to insects, plants, and animals and are also present in human blood serum (Gohel et al. 2006).

Modern biotechnology has raised the development of bioprocesses to use microbes to produce value-added bio-chemicals like enzymes (Yan and Fong 2015). Chitinolytic microorganisms play an indispensable biogeochemical role in chitin bioprocessing (Ilangumaran et al. 2017). Chitinase-producing microorganisms exhibit their wide range of distribution in the environment. Not only they are present in extreme habitat like Antarctic soil, hot spring, and soda lake, but also their attendance was observed from crustaceans' waste to gut system, rhizospheric soil, and endophytic domains. These workhorses of the chitinase production company are both the eukaryotic and prokaryotic types of microorganisms. Chitinolytic fungi comprise 25–60% of the entire mold fungi, but their figure is inferior to the digit of bacteria (Brzezinska et al. 2014). The majority of the fungi belong to *Ascomycota*, whereas in bacteria, *Proteobacteria* are dominant over *Firmicutes*, *Actinobacteria*, and *Bacteroidetes* (Fig. 18.1).

Apart from the chitinase-producing capability of fungi, it is also responsible for causing various plant diseases. Plant diseases cause massive loss to the plant population together with economically important crop plants, causing misery to human beings (late blight of potato by *Phytophthora infestans* and brown spot of rice by *Helminthosporium oryzae* lead to Irish and Bengal famines, respectively) (Agrios 2005). Fungal phytopathogens are the serious intimidations to the commercial crops like cereals, potatoes, vines, fruits, and vegetables and are orthodoxly demolished by chemical fungicides. But the extensive uses of chemical fungicides are presumed to be lethal for the beneficial insects and microorganisms in the habitat soil and invade the food chain through biomagnification, leading to metabolic disorders, massive mutation, and carcinogenic effect on human beings. But modern approaches like biological control through biomolecules like chitinases for aiding sustainable agriculture give a substitute environment-friendly policy for monitoring phytopathogens like insects, fungi, and nematodes (Gaurav et al. 2017; Brzezinska et al. 2014).

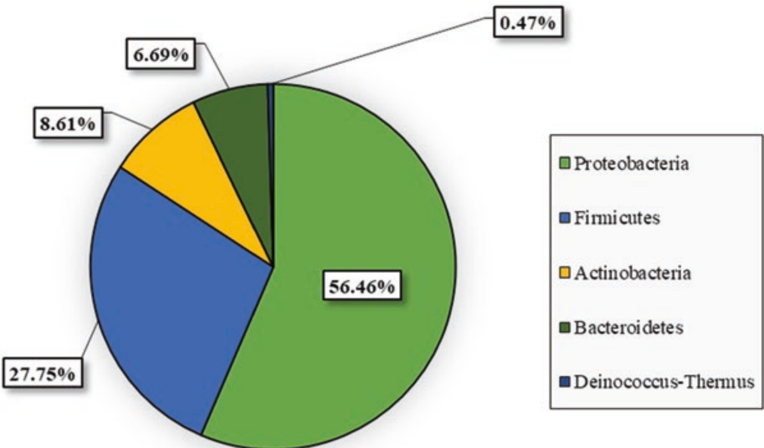

Fig. 18.1 Abundance of chitinase-producing culturable bacteria

So, microorganisms from diversified natural resources with chitinolytic activity can open a new arena in biotechnological approaches as a "green fungicide" or "green insecticide" or as a whole "green pesticide" and also can be a treasure box for human welfare as it may replace the use of chemical fungicide and insecticide.

18.2 Diversity of Culturable Chitinase-Producing Bacteria

Microorganisms utilize composite chitin molecule as carbon and energy source by hydrolyzing it into simple sugars known as the chitinase producers (Gaurav et al. 2017). Several natural resources are used for isolation of chitinase-producing bacteria and fungi. Such natural resources are like soil, water, shrimp shell waste, crab cell waste, fishing fields, seafood-processing industries, plant endophytes, and gut systems. The soil resources reflect great variations like agricultural, rhizospheric, mangrove, and Antarctic soils. The water resources are like hot spring, soda lake, Lonar lake, freshwater lake, marine water, and shrimp-cultivating ponds. Among the gut systems, both the vertebrate (fish and bat) and invertebrate (insect, earthworm) are explored. Chitinolytic bacterial flora consists of both the Gram-positive and Gram-negative types with respect to all the isolated fields. Among the reported culturable bacterial diversity, *Proteobacteria* is the predominant one (56.46%) followed by *Firmicutes* (27.75%), *Actinobacteria* (8.61%), *Bacteroidetes* (6.69%), and *Deinococcus-Thermus* (0.47%) (Fig. 18.1). Culturable microorganisms possess chitinase production with habitat specificity and are listed in Table 18.1.

Table 18.1 Chitinase-producing soil bacteria

Organism	Habitat	Phylum	References
Chromobacterium sp.	Cultivation soil	*Proteobacteria*	Han et al. (2018)
Streptomyces samsunensis UAE1	Mango rhizospheric soil	*Actinobacteria*	Kamil et al. (2018)
Micromonospora tulbaghiae UAE1		*Actinobacteria*	
Stenotrophomonas maltophilia	Agricultural rhizospheric soil	*Proteobacteria*	Shaikh et al. (2018)
Pseudomonas sp.		*Proteobacteria*	
Alcaligenes sp.		*Proteobacteria*	
Bacillus sp.	Saline soil	*Firmicutes*	Jafari et al. (2018)
Paenibacillus sp.		*Firmicutes*	
Staphylococcus sp.		*Firmicutes*	
Bacillus sp.	Tea rhizospheric soil	*Firmicutes*	Vandana et al. (2018)
Pseudomonas sp.		*Proteobacteria*	
Bacillus pumilus RST25	Shellfish-processing industrial soil	*Firmicutes*	Gaurav et al. (2017)
Pseudomonas sp.	Avocado field soil	*Proteobacteria*	Vida et al. (2017)
Serratia sp.		*Proteobacteria*	
Stenotrophomonas sp.		*Proteobacteria*	
Bacillus sp. SJ-5	Soybean rhizospheric soil	*Firmicutes*	Jain et al. (2017)
Enterobacter sp.	Soil sample	*Proteobacteria*	Ong et al. (2017)
Zymomonas sp.		*Proteobacteria*	
Streptomyces mexicanus	Agricultural and industrial soils	*Actinobacteria*	Das et al. (2017)
S. albidoflavus		*Actinobacteria*	
Pedobacter sp. PR-M6	Decayed mushroom soil	*Bacteroidetes*	Song et al. (2017)
Streptomyces sp.	Vineyard soil	*Actinobacteria*	Ilangumaran et al. (2017)
Pseudomonas putida	Rhizospheric soil	*Proteobacteria*	Keshavarz-Tohid et al. (2017)
Pseudomonas fluorescens	Soil isolate	*Proteobacteria*	Alhasawi and Appanna (2017)
Loktanella fryxellensis	Antarctic soil	*Proteobacteria*	Shivaji et al. (2017)
L. salsilacus		*Proteobacteria*	
L. vestfoldensis		*Proteobacteria*	
Pseudorhodobacter antarcticus		*Proteobacteria*	
P. psychrotolerans		*Proteobacteria*	
Robiginitomaculum antarcticum		*Proteobacteria*	
Roseicitreum antarcticum		*Proteobacteria*	
R. antarcticus		*Proteobacteria*	
Sphingomonas aerolata		*Proteobacteria*	
S. aurantiaca		*Proteobacteria*	

(continued)

Table 18.1 (continued)

Organism	Habitat	Phylum	References
S. faeni		*Proteobacteria*	
Alteromonas stellipolaris		*Proteobacteria*	
Glaciecola polaris		*Proteobacteria*	
Granulosicoccus antarcticus		*Proteobacteria*	
Lysobacter oligotrophicus		*Proteobacteria*	
Marinomonas polaris		*Proteobacteria*	
Shewanella livingstonensis		*Proteobacteria*	
S. vesiculosa		*Proteobacteria*	
Antarcticimonas flava		*Bacteroidetes*	
Cellulophaga algicola		*Bacteroidetes*	
Flavobacterium collinsense	Antarctic soil	*Bacteroidetes*	Shivaji et al. (2017)
Gelidibacter gilvus		*Bacteroidetes*	
Leeuwenhoekiella aequorea		*Bacteroidetes*	
Muricauda antarctica		*Bacteroidetes*	
Pedobacter ardleyensis		*Bacteroidetes*	
Polaribacter sejongensis		*Bacteroidetes*	
Salegentibacter salegens		*Bacteroidetes*	
Exiguobacterium soli		*Firmicutes*	
Paenibacillus cookii		*Firmicutes*	
Planococcus maitriensis		*Firmicutes*	
Psychrosinus fermentans		*Firmicutes*	
Leifsonia rubra		*Actinobacteria*	
Marisediminicola antarctica		*Actinobacteria*	
Pseudonocardia antarctica		*Actinobacteria*	
Deinococcus frigens		*Deinococcus-Thermus*	
Bacillus pumilus		*Firmicutes*	Rishad and Jisha (2016)
B. aerophilus		*Firmicutes*	
Pseudomonas plecoglossicida	Mangrove soil	*Proteobacteria*	
Achromobacter insolitus		*Proteobacteria*	
Lysinibacillus fusiformis		*Firmicutes*	
Bacillus sp.	Rhizospheric soil	*Firmicutes*	Thakkar et al. (2016)
Aeromonas hydrophila	Rhizospheric soil	*Proteobacteria*	Kuddus and Ahmad (2013)
A. punctata	Fish processing effluent	*Proteobacteria*	
Streptomyces rimosus	Agricultural soil	*Actinobacteria*	Brzezinska et al. (2013)

(continued)

Table 18.1 (continued)

Organism	Habitat	Phylum	References
Stenotrophomonas maltophilia	Rhizospheric soil	*Proteobacteria*	Jankiewicz et al. (2012)
Serratia sp.	Rhizosphere of agronomic plant	*Proteobacteria*	Someya et al. (2011)
Stenotrophomonas sp.		*Proteobacteria*	
Lysobacter sp.		*Proteobacteria*	
Mitsuaria sp.		*Proteobacteria*	
Paenibacillus sp.		*Firmicutes*	
Bacillus sp.		*Firmicutes*	
Erwinia sp.		*Proteobacteria*	
Aeromonas sp.		*Proteobacteria*	
Pseudomonas sp.		*Proteobacteria*	
Achromobacter sp.		*Proteobacteria*	
Flavobacterium sp.		*Bacteroidetes*	
Microbacterium sp.		*Actinobacteria*	
Bacillus pumilus	Soil sample from various locations in Iran	*Firmicutes*	Tasharrofi et al. (2011)
Serratia sp.	Rhizospheric soil of rice fields	*Proteobacteria*	Amin et al. (2011)
Pseudomonas sp.		*Proteobacteria*	
Bacillus cereus	Rhizospheric soil of pepper	*Firmicutes*	Mubarik et al. (2010)
Bacillus licheniformis	Rhizospheric soil of maize, wheat, and rice	*Firmicutes*	Kamil et al. (2007)
B. thuringiensis		*Firmicutes*	
Stenotrophomonas maltophilia		*Proteobacteria*	
Bacillus sp.	Soil samples from Youngduck, South Korea	*Firmicutes*	Joo et al. (1996)

18.2.1 Chitinase-Producing Bacteria Isolated from Soil

Reports regarding chitinase-producing soil bacteria are studied in detail so far. A list of soil bacteria with chitinolytic activity are given in Table 18.1. Among the reported bacterial diversity, *Proteobacteria* is the dominant group (52.27%) over the *Firmicutes* (22.72%), *Bacteroidetes* (12.5%), *Actinobacteria* (11.36%), and *Deinococcus-Thermus* (1.13%) (Fig. 18.2).

18.2.2 Chitinase-Producing Bacteria Isolated from Different Water Bodies

Chitinase-producing bacteria are also reported from various water bodies such as shrimp ponds, marine water, Lonar lake, hot spring, and moat water. Among them, shrimp-cultivating ponds are the potent container of the chitinolytic bacteria. The

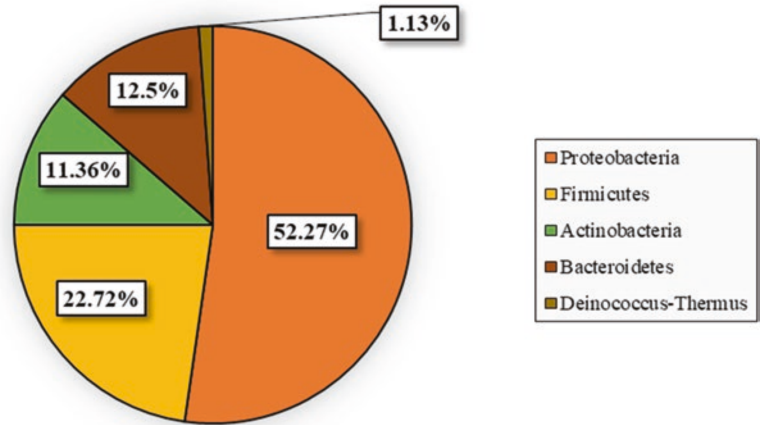

Fig. 18.2 Diversity of chitinase-producing soil bacteria

Table 18.2 Waterborne chitinolytic bacteria

Water body	Organism	Phylum	References
Marine water	*Paenibacillus* sp. AD	*Firmicutes*	Kumar et al. (2018)
Freshwater lake	*Andreprevotia lacus*	*Proteobacteria*	Tran et al. (2018)
	Brevibacillus brevis	*Firmicutes*	
	Aeromonas hydrophila	*Proteobacteria*	
	A. salmonicida	*Proteobacteria*	
	Serratia plymuthica	*Proteobacteria*	
Irrigation well water	*Pseudomonas* sp.	*Proteobacteria*	Tabli et al. (2018)
	Serratia sp.	*Proteobacteria*	
Marine water	*Bacillus cereus*	*Firmicutes*	Ravikumar and Perinbam (2016)
Hot spring	*Paenibacillus* sp.	*Firmicutes*	Chrisnasari et al. (2016)
Shrimp pond	*Vibrio alginolyticus*	*Proteobacteria*	Vincy et al. (2014)
Moat water	*Chitiniphilus shinanonensis*	*Proteobacteria*	Huang et al. (2012)
Lonar lake	*Streptomyces* sp.	*Actinobacteria*	Bansode and Bajekal (2006)
	Nocardia sp.	*Actinobacteria*	
	Bacillus sp.	*Firmicutes*	

list of chitinase-producing bacteria isolated from different water bodies are presented in Table 18.2. In the middle of all reported bacterial variations from the different water bodies, *Proteobacteria* is the mostly rich group of bacteria (57.14%) followed by *Firmicutes* (28.57%) and *Actinobacteria* (14.28%) (Fig. 18.3).

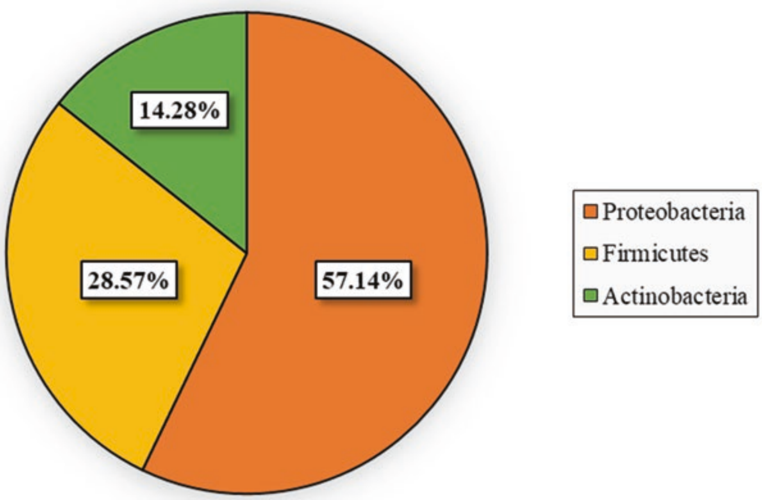

Fig. 18.3 Chitinase-producing bacteria isolated from water bodies

Table 18.3 Chitinolytic bacteria isolated from crab cell waste

Organism	Phylum	References
Vibrio aestuarianus	*Proteobacteria*	Anuradha and Revathi (2013)
Flavobacterium sp.	*Bacteroidetes*	
Shewanella sp.	*Proteobacteria*	
Exiguobacterium sp.	*Firmicutes*	
Aeromonas sp.	*Proteobacteria*	Ahmadi et al. (2008)

18.2.3 Chitinase-Producing Bacteria Isolated from Crab Shell Waste

Crab cells are made up of chitin. Therefore, promising chitinase-producing bacteria can be isolated from these wastes. Reports regarding the chitinolytic bacteria from crab cell wastes are recorded in Table 18.3. Bacterial diversity in this area is commanded by *Proteobacteria* (60%), and the rest of the representatives are from *Firmicutes* (20%) and *Bacteroidetes* (20%) (Fig. 18.4).

18.2.4 Chitinase-Producing Bacteria Isolated from Shrimp Shell Waste

Shrimp shell wastes are the major sources of chitin as they are made up of chitinous exoskeleton. Reports regarding the bacteria isolated from the shrimp shell waste are enlisted in Table 18.4. Data regarding the bacterial diversity from the shrimp shell

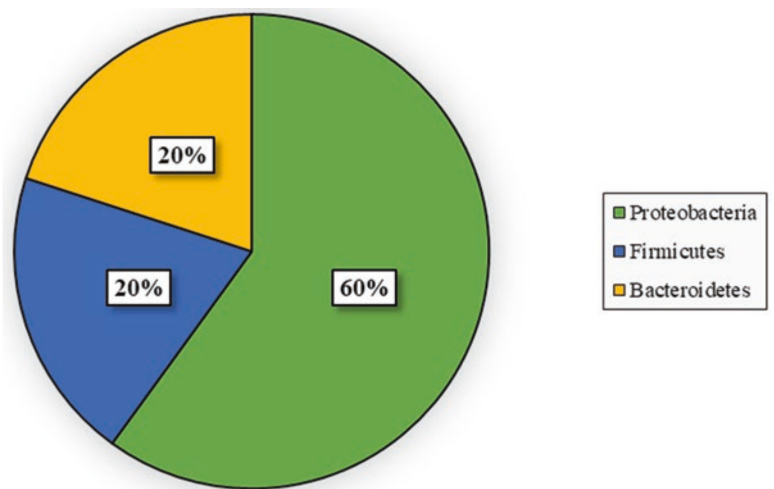

Fig. 18.4 Chitinase-producing bacteria isolated from crab cell waste

waste are dominated by *Proteobacteria* (66.66%) over the *Actinobacteria* (16.66%) and *Firmicutes* (16.66%), as shown in Fig. 18.5.

18.2.5 Chitinase-Producing Endophytic Bacteria

Endophytic bacteria with chitinase production ability are reported from economically important crop plants like potato, maize, and brassica. A list is given in the Table 18.5. In this area, most of the chitinolytic bacteria are from the *Proteobacteria* (40%), *Firmicutes* (40%), and *Actinobacteria* (20%) (Fig. 18.6).

18.2.6 Chitinase-Producing Gut Bacteria

Chitinase production by the gut bacteria is reported among the invertebrates and vertebrates. Among the invertebrates, insect and earthworm are the only reports where chitinolytic symbiotic gut microbes are observed (Tables 18.7 and 18.8). Fish and bat are the two vertebrates where chitinase-producing gut bacteria (Tables 18.6 and 18.9) are studied so far. Here, the reported gut bacteria are listed in Table 18.6.

Table 18.4 Chitinolytic bacteria isolated from shrimp shell waste

Organism	Phylum	References
Paenibacillus elgii TS 33	*Firmicutes*	Tariq et al. (2017)
Acinetobacter johnsonii	*Proteobacteria*	Setia and Suharjono (2015)
Bacillus amyloliquefaciens	*Proteobacteria*	
Aeromonas hydrophila	*Proteobacteria*	Halder et al. (2013)
Streptomyces sp.	*Actinobacteria*	Thiagarajan et al. (2011)
Aeromonas sp.	*Proteobacteria*	Ahmadi et al. (2008)

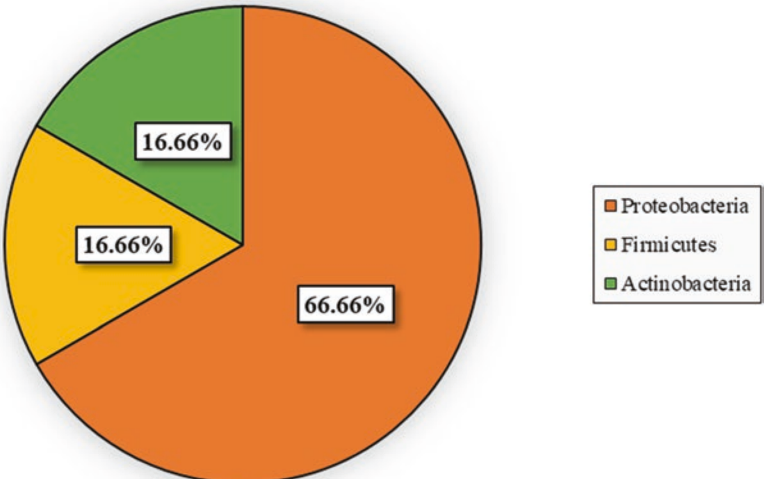

Fig. 18.5 Chitinolytic bacteria from shrimp cell waste

Table 18.5 Endophytic chitinolytic bacteria

Organism	Phylum	Plant	References
Rhizobium sp.	*Proteobacteria*	*Zea mays*	Patel and Archana (2017)
Arthrobacter sp.	*Actinobacteria*	Roots of *Brassica rapa*	Padder et al. (2017)
Rhizobium sp.	*Proteobacteria*		
Bacillus sp.	*Firmicutes*		
Bacillus licheniformis	*Firmicutes*	Stem tissue of *Solanum tuberosum*	Aounallah et al. (2017)

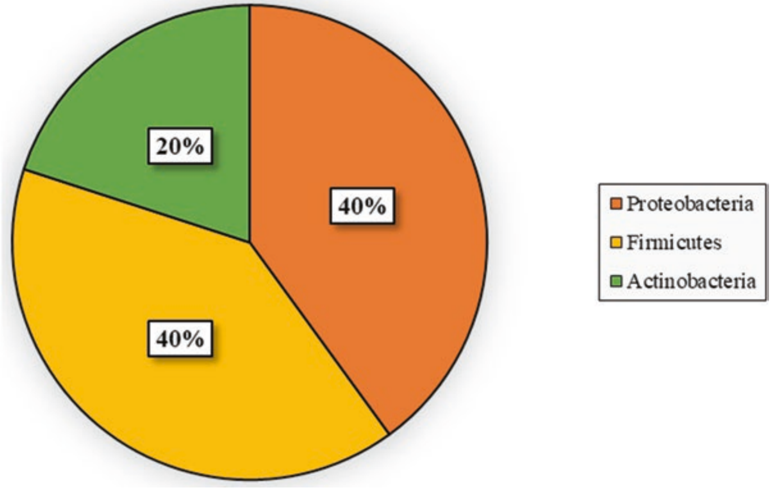

Fig. 18.6 Chitinolytic endophytic bacteria

18.2.6.1 Chitinase-Producing Fish Gut Bacteria (Table 18.6)

Table 18.6 Chitinolytic fish gut bacteria

Organism	Phylum	Fish	References
Pseudomonas sp. SSPZ11	*Proteobacteria*	*Rastrelliger kanagurt*	Thomas et al. (2018)
Exiguobacterium sp. SSPZ15	*Firmicutes*	*Catla catla*	
Vibrio sp.	*Proteobacteria*	*Paralichthys adspersus*	Leiva et al. (2017)
Bacillus sp.	*Firmicutes*		
Photobacterium sp.	*Proteobacteria*		
Staphylococcus sp.	*Firmicutes*		
Carnobacterium sp.	*Firmicutes*		
Exiguobacterium sp.	*Firmicutes*		
Klebsiella sp.	*Proteobacteria*		
Arthrobacter sp.	*Actinobacteria*		
Raoultella sp.	*Proteobacteria*		
Kluyvera sp.	*Proteobacteria*		
Myroides sp.	*Bacteroidetes*		
Streptococcus sp.	*Firmicutes*		
Vagococcus sp.	*Firmicutes*		
Staphylococcus sp.	*Firmicutes*		
Acinetobacter sp.	*Proteobacteria*		
Psychrobacter sp.	*Proteobacteria*		
Lactobacillus sp.	*Firmicutes*		
Weissella sp.	*Firmicutes*		
Lactococcus sp.	*Firmicutes*		
Bacillus cereus	*Firmicutes*	*Clarias gariepinus*	Ajayi et al. (2016)
Bacillus aryabhattai	*Firmicutes*	*Clarias batrachus*	Dey et al. (2016)
B. flexus	*Firmicutes*		
B. cereus	*Firmicutes*		

(continued)

Table 18.6 (continued)

Organism	Phylum	Fish	References
Bacillus pumilus	*Firmicutes*	*Labeo rohita*	Banerjee et al. (2015)
B. flexus	*Firmicutes*	*Catla catla*	
		Cirrhinus mrigala	
Pseudomonas sp.	*Proteobacteria*	*Gadus morhua*	Lazado et al. (2012)
Psychrobacter sp.	*Proteobacteria*		
Acinetobacter johnsonii	*Proteobacteria*	*Salmo salar*	Askarian et al. (2012)
Acinetobacter sp.	*Proteobacteria*		
Agrococcus baldri	*Actinobacteria*		
Bacillus cereus	*Firmicutes*		
B. thuringiensis	*Firmicutes*		
B. subtilis	*Firmicutes*		
Bacillus sp.	*Firmicutes*		
Carnobacterium sp.	*Firmicutes*		
Staphylococcus equorum	*Firmicutes*		
Staphylococcus sp.	*Firmicutes*		
Vibrio fischeri	*Proteobacteria*	*Paralichthys olivaceus*	Sugita and Ito (2006)
V. scophthalmi	*Proteobacteria*		
V. ichthyoenteri	*Proteobacteria*		
V. carchariae	*Proteobacteria*		
V. harveyi	*Proteobacteria*		
V. scophthalmi	*Proteobacteria*		
Ferrimonas balearica	*Proteobacteria*	*Canthigaster rivulata*	Itoi et al. (2006)
Pseudoalteromonas piscicida	*Proteobacteria*	*Ditrema temmincki*	
Grimontia hollisae	*Proteobacteria*	*G. punctate*	
Photobacterium damselae	*Proteobacteria*	*Gonnistius zonatus*	
P. leiognathi	*Proteobacteria*		
P. lipolyticum	*Proteobacteria*		
P. phosphoreum	*Proteobacteria*	*Gymnothorax kidako*	
P. rosenbergii	*Proteobacteria*		
Vibrio chagasii	*Proteobacteria*	*Microcanthus strigatus*	
V. fischeri	*Proteobacteria*		
V. fortis	*Proteobacteria*		
V. gallicus	*Proteobacteria*		
V. harveyi	*Proteobacteria*		
V. natriegens	*Proteobacteria*	*Parajulis poecilepterus*	
V. nigripulchritudo	*Proteobacteria*		
V. ordalii	*Proteobacteria*		
V. parahaemolyticus	*Proteobacteria*		
V. pomeroyi	*Proteobacteria*		
V. ponticus	*Proteobacteria*	*Pseudocaranx dentex*	
V. proteolyticus	*Proteobacteria*		
V. rumoiensis	*Proteobacteria*		
V. shilonii	*Proteobacteria*	*Girella leonina*	
V. tasmaniensis	*Proteobacteria*		
V. tubiashii	*Proteobacteria*		

18.2.6.2 Chitinase-Producing Insect Gut Bacteria (Table 18.7)

Table 18.7 Chitinolytic insect gut bacteria

Organism	Phylum	Insect	References
Cellulomonas macrotermitis	*Actinobacteria*	*Macrotermes barneyi*	Sun et al. (2018)
Pseudomonas sp.	*Proteobacteria*	*Plutella xylostella*	Indiragandhi et al. (2007)
Stenotrophomonas sp.	*Proteobacteria*		
Acinetobacter sp.	*Proteobacteria*		
Serratia marcescens	*Proteobacteria*		

18.2.6.3 Chitinase-Producing Earthworm Gut Bacteria (Table 18.8)

Table 18.8 Chitinolytic earthworm bacteria

Organism	Phylum	Earthworm	Reference
Pseudomonas stutzeri EGB3	Proteobacteria	*Eisenia foetida*	Prasanna et al. (2014)

18.2.6.4 Chitinase-Producing Gut Bacteria of Bat (Table 18.9)

Table 18.9 Chitinolytic bat gut bacteria

Organism	Phylum	Bat	References
Serratia liquefaciens	*Proteobacteria*	*Myotis lucifugus*	Whitaker et al. (2004)
S. marcescens	*Proteobacteria*	*M. septentrionalis*	
Bacillus coagulans	*Firmicutes*		
B. thuringiensis	*Firmicutes*		
B. cereus	*Firmicutes*		
Enterobacter agglomerans	*Proteobacteria*		
E. aerogenes	*Proteobacteria*		
E. cloacae	*Proteobacteria*		
Hafnia alvei	*Proteobacteria*		
Citrobacter amelonaticus	*Proteobacteria*		

Chitinase-producing bacteria from different natural resources are stated in this chapter. There are many reports available in regard to soil and water. Reports in relation to shrimp shell waste and crab cell waste are plenty, but gut bacterial reports for chitinase production are limited only in two groups, i.e., insect and earthworm

(invertebrates) and fish and bat members (vertebrates). There are vast resources of unexplored fields in relation to chitinase-producing gut microbes. Therefore, gut microorganisms possessing chitinolytic activity can be a hidden tool toward the biotechnological approaches (Figs. 18.7, 18.8, 18.9 and 18.10).

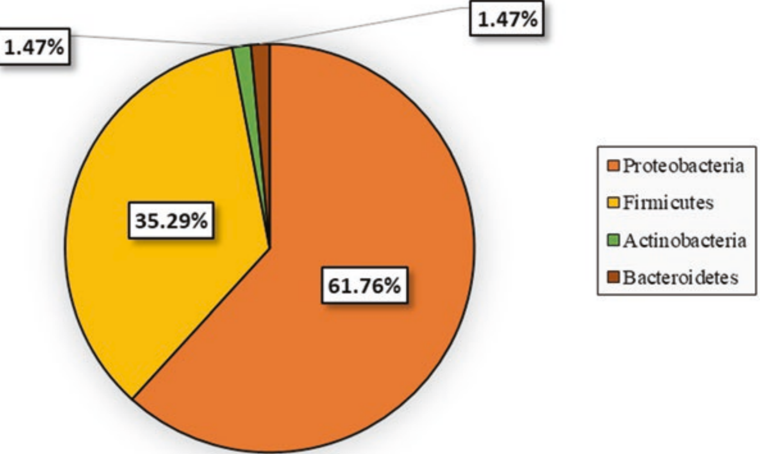

Fig. 18.7 Chitinolytic fish gut bacteria

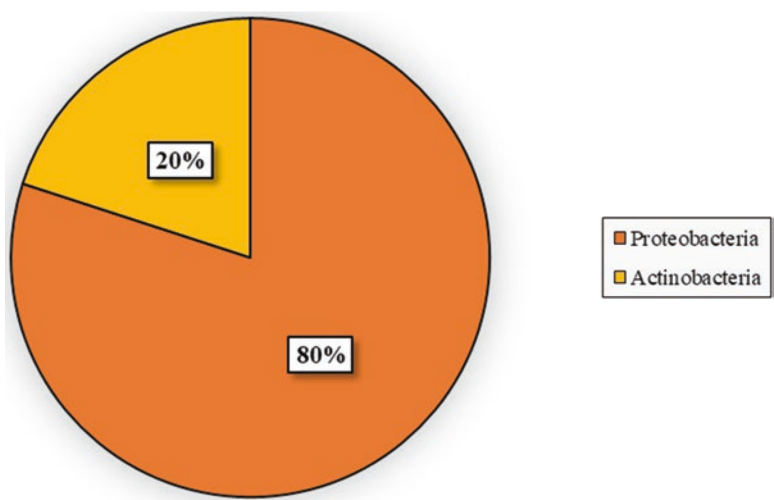

Fig. 18.8 Chitinolytic insect gut bacteria

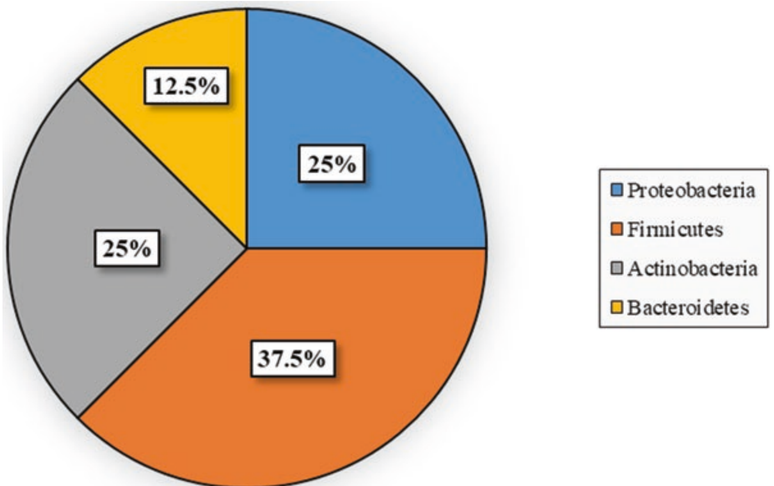

Fig. 18.9 Chitinolytic earthworm gut bacteria

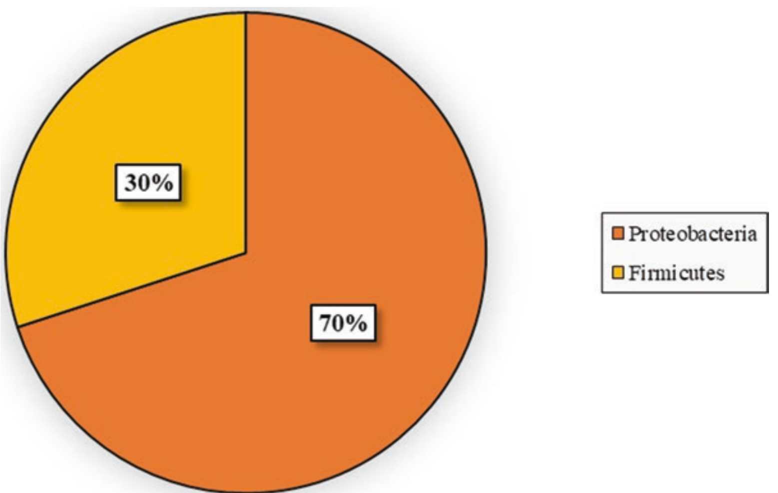

Fig. 18.10 Chitinolytic bat gut bacteria

18.3 Their Role in the Habitat

Microorganisms which utilize merged chitin molecule as carbon and energy source by hydrolyzing it into simple sugars are known as the chitinase producers (Gaurav et al. 2017). Their wide-ranging abundance has already been stated earlier. Chitinolytic microbes can be isolated from the habitats on the basis of availability of their food material like chitin. Such habitats cover from shrimp shell waste area and crab shell dumping zone to soil, water, gut environments, and so on. Microbial

residency on these types of locale plays an indispensable role to simplify the rigid chitin which subsequently produces oligomers and monomers, and these products cause several beneficial benefits toward the residing environment, chiefly as the biofertilizing, biocontrolling, and biowaste managing agents.

Among the natural resources, crustacean biowastes exclusively shrimp and crab shells have the maximum chitin content up to 60% (Chakrabarti 2002; Wang et al. 2006; Kandra et al. 2012). Annually, around 10^{11} tons of chitinous ingredients are produced in the aquatic environment, but there is no considerable addition of chitin in the ocean sediments as the chitinolytic microorganisms in the aquatic ecosystem basically degrade them (Ghorbel-Bellaaj et al. 2012; Halder et al. 2012). So, the microbial population belonging to these habitats like marine water, shrimp shell waste, and crab shell waste exhibits a significant chitin-reducing activity as they utilize these biowastes as nutritional resources. Evidences are also available in support of the bacterial type isolated from these habitats (Tables 18.2, 18.3, and 18.4). Microbial residents in such type of habitats are also serving as an environmentally autoregulated biowaste management agent. Marine microorganisms have established inimitable metabolic and physiological abilities to harvest novel metabolites which are not often existing in microbes of terrestrial origin. Away from their biorecycling capability, some marine bacteria have a good potential for the control of fungal phytopathogens and mycotoxins (Kong 2018).

Reports concerning the chitinase-producing microorganisms isolated from the variable soil environments are numerous and listed in Table 18.1. The presence of such kind of microbes plays several advantageous characters in that type of soil atmosphere. From antifungal assets are through chitinase production to plant growth-promoting properties like phosphate and zinc solubilization ability, indole 3-acetic acid and siderophore production, seed germination enhancing ability, etc. (Sarbadhikary and Mandal 2017; Kejela et al. 2017; Patel and Arcahna 2017; Adhikari et al. 2017). In the current scenario, the participation of microbial inoculants as biofertilizers and biocontrol agents in the agriculture industry has been growing noticeably. Microbial inoculants are favored to reduce environmental toxicity instigated by chemicals and pesticides.

In the case of gut ambience, the presence of such type of microorganisms strictly depends upon the food habit of the host because they take part in the host's digestion and nutritive processes. Microbes that degrade the dietary compounds can retain, proliferate, and establish symbiosis, and the others that are unable to degrade are washed out (Banerjee et al. 2017). In the later part of this endeavor, it can be observed that several reports are available related to gut microbes of insect, earthworm, fish, and bat that can hydrolyze chitin. These hosts are the consumers of chitinous materials, and it can be assumed that these gut microbes play a role in their digestion, vitamin synthesis, and antifungal activity with their chitinolytic efficiency (Dillon and Dillon 2004; Genta et al. 2006).

So, in this framework, the role of the chitinase producers in their habitats stands with a great biotechnological importance for modern-day sustainable agriculture, which leads to a pronounced human welfare phenomenon by replacing ecotoxic chemical fertilizers, fungicides, and pesticides.

18.4 Potential Applications

Microbial enzymes are relatively more stable than corresponding enzymes derived from plants or animals (Wiseman 1995). Enzymes of microbial origin have been used in various industries for many centuries. Enzymes from microbial sources are widely used in industrial processes mainly because of their low cost, large productivity, vast availability, chemical stability, and flexibility (Banerjee et al. 2016), and bacterial chitinases are such kind of biomolecules. Chitinase enzyme has received increased attention due to its wide range of biotechnological applications, especially in agriculture for biocontrol of phytopathogenic fungi and harmful insects (Kuddus and Ahmad 2013). Chitinases are with immense importance in the biotechnology and bioprocessing ranges; because of their versatile potentiality as pesticide (against fungi, insects, and nematodes), they induce plant disease resistance, alternative petroleum feedstock, waste water management, marine by-products treatment (shrimp shell waste and sea food degradation), pharmaceutical industry activities (chitosaccharides), protoplast isolation from fungi and yeast, and preparation of single-cell protein (Kumar et al. 2018; Mao et al. 2017; Ilangumaran et al. 2017; Honda et al. 2017; Wang and Liang 2017; Aggarwal et al. 2015; Brzezinska et al. 2014; Halder et al. 2013; Mubarik et al. 2010).

18.4.1 Induce Plant Defense System

Biocontrol activities and plant growth-promoting potentialities are not only synchronized by the bacterial chitinolytic property but also obtained by the derivatives of chitin molecules. Their operational machineries are the outcome in direct antimicrobial responsibilities, stimulation of plant defense responses, and plant metabolic activity (El Hadrami et al. 2010; Ramírez et al. 2010). Chitosan has the capability to prevent the growth of a variety of bacteria and fungi (Rabea et al. 2003; El Hadrami et al. 2010; Xia et al. 2011; Sharp 2013). The antimicrobial potentiality of chitosan is known for its cationic features, which disrupt potassium signaling cascade in pathogens. Furthermore, chitosan interrupts membrane integrity of vacuoles and endomembrane organelles in fungal pathogens (Rabea et al. 2003; Sharp 2013). One such example was investigated by O'Herlihy et al. (2003) where chitosan exhibits the inhibitory activity against *Phytophthora capsici* and *P. infestans*. Another improvised nanotechnology-based work has been revealed by Chandra et al. (2015) where the chitosan nanoparticles (CNP) are capable of inducing and augmenting immune response in plants. CNP-treated leaves of *Camellia sinensis* produced substantial progress in the plant's innate immune response by the induction of defense enzyme activity, upregulation of defense-related genes including that of several antioxidant enzymes, and elevation of the levels of total phenolics (Fig. 18.11).

Chitin oligosaccharides perform as pathogen-associated molecular patterns (PAMPs) due to their structural resemblance to the ingredients of pathogen cell wall in various plant pathosystems. PAMPs are accepted by host transmembrane pattern recognition receptors (PRRs), which signal defense corridors of induced systemic

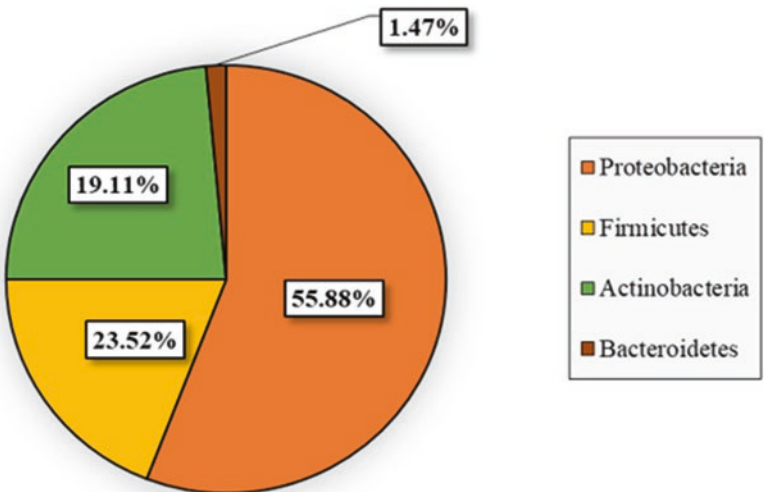

Fig. 18.11 Antifungal bacterial diversity

resistance (ISR) and systemic acquired resistance (SAR) (Eckardt 2008; Zipfel 2009). As a result, when real pathogen occurrence happens, the plant disease resistance mechanisms deliberate boosted protection against it. Thus, chitin derivatives attained from microbial degradation of crustaceans shells can be applied as elicitors of innate and systemic immune responses in plants (Benhamou 1996; Jones and Dangl 2006). The chitinous extracts assembled from microbial degradation were applied to induce disease resistance in *Arabidopsis thaliana* against *Pseudomonas syringae* pv. tomato DC3000 and *Botrytis cinerea* (Ilangumaran et al. 2017).

18.4.2 Antifungal Activity of Bacterial Chitinase

Awareness in biological control has amplified over the past decades. The necessity for the replacements of chemical fungicides arises because of their penetration into the food chain which leads to the human health hazard and establishes resistant phytopathogens and also accelerates environmental contamination in parallel. Recently, biological control has been dedicated on bacteria-producing mycolytic enzymes, exclusively chitinases, recognized to hydrolyze chitin, a key element of fungal cell walls. In this context, antagonistic bacterial chitinases provide an environmentally sound substitute to synthetic chemicals because of their perceived safety and inferior environmental impact. Biological control policies have turned into an imperative attitude for aiding sustainable agriculture (Brzezinska et al. 2014; Berg and Hallmann 2006). Commercial biocontrol representatives mainly belong to the spore-forming bacteria because of their durability in a diversity of formulations and field environments for an extended period even under adverse situations (Subbanna et al. 2018).

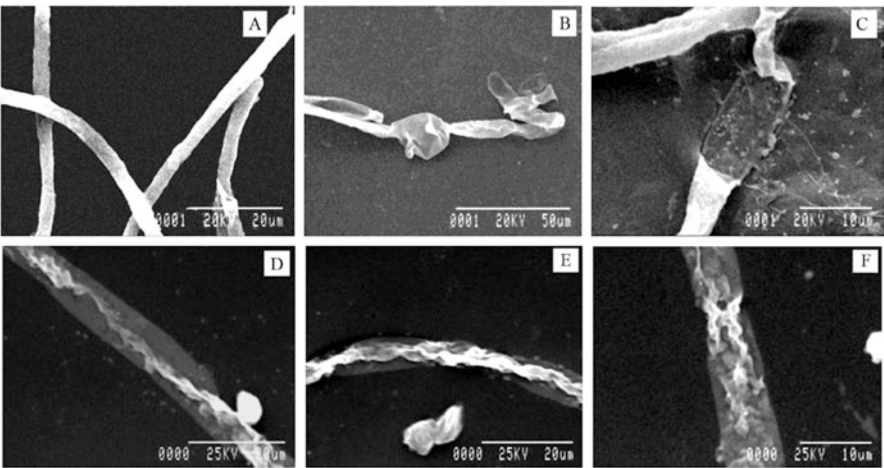

Fig. 18.12 Degradation of fungal (*Rhizopus stolonifer*) cell wall by chitinase-producing bacterial strains (Ghosh et al. 2015)

The mainstream of pathogenic fungi comprises chitin and β-(1,3) glucan in their cell walls (Bartnicki-Garcia 1968), and disbanding or disruption of these organizational polymers has negative consequences over the growth and differentiation of fungi (Poulose 1992). One of the key antagonist apparatuses used by the biocontrol agents for these types of phytopathogens is the enzymatic disintegration of cell walls heading to leakage of fungal protoplasm (Lim et al. 1991; Kim and Chung 2004). Cell wall-degrading enzymes, especially chitinolytic enzyme-producing biocontrolling bacteria, are able to effectively control plant pathogenic fungi in this way (Broglie et al. 1991; Ordentlich et al. 1988) (Fig. 18.12). Abilities of bacteria to produce antifungal chitinase are widely known (Table 18.10), and the majority of biocontrolling bacteria belong to *Proteobacteria* (55.88%), *Firmicutes* (23.52%), *Actinobacteria* (19.11%), and *Bacteroidetes* (1.47%).

18.4.3 Insecticidal Activity of Bacterial Chitinase

Insect infestation is a major issue of many agronomic crops. Insects attack more than 500 plant species belonging to 63 plant families. Insects are the vector of plant virus member especially of the geminivirus group. Some diseases associated with the whitefly are lettuce necrotic yellows, irregular ripening of tomato, silver leaf of squash, cotton leaf curl, tobacco leaf curl, and cassava mosaic. Meanwhile, chitin-scavenging enzymes are applied to renovate chitin-holding raw material into biotechnologically serviceable apparatuses; they are a significant concern of chemical and pharmaceutical activities (Aggarwal et al. 2015).

In insects, the dynamic configurations such as exoskeleton, appendages, peritrophic membrane, etc. are made up of chitin as a chief structural element. Therefore, the growth and development are intensely administrated by building and

Table 18.10 Antifungal activity of bacterial chitinases

Bacteria	Phylum	Antagonistic against	References
Chromobacterium sp.	*Proteobacteria*	*Cylindrocarpon destructans*	Han et al. (2018)
Streptomyces samsunensis UAE1	*Actinobacteria*	*Lasiodiplodia theobromae*	Kamil et al. (2018)
Micromonospora tulbaghiae UAE1	*Actinobacteria*		
Bacillus sp.	*Firmicutes*	*Rhizoctonia solani*	Vandana et al. (2018)
Pseudomonas sp.	*Proteobacteria*	*Corticium invisum*	
		Fomes lamanensis	
Aeromonas salmonicida SWSY-1.411	*Proteobacteria*	*Trichoderma reesei*	Tran et al. (2018)
A. salmonicida SWSY-1.31	*Proteobacteria*		
Serratia plymuthica SWSY3.47	*Proteobacteria*		
Pseudomonas sp.	*Proteobacteria*	*Aspergillus niger*	Tabli et al. (2018)
Serratia sp.	*Proteobacteria*	*Botrytis cinerea*	
		Pythium aphanidermatum	
Corallococcus sp. EGB	*Proteobacteria*	*Verticillium dahliae*	Li et al. (2017)
		Fusarium oxysporum	
		Ustilaginoidea virens	
Pseudomonas sp.	*Proteobacteria*	*Rosellinia necatrix*	Vida et al. (2017)
Serratia sp.	*Proteobacteria*		
Stenotrophomonas sp.	*Proteobacteria*		
Bacillus sp. SJ-5	*Firmicutes*	*Rhizoctonia solani*	Jain et al. (2017)
		Fusarium oxysporum	
Bacillus pumilus RST25	*Firmicutes*	*Fusarium solani*	Gaurav et al. (2017)
		Aspergillus niger	
Paenibacillus elgii.	*Firmicutes*	*Fusarium solani*	Tariq et al. (2017)
		Aspergillus parasiticus	
		A. fumigates	
Pedobacter sp. PR-M6	*Bacteroidetes*	*Rhizoctonia solani*	Song et al. (2017)
		Botrytis cinerea	
Pseudomonas sp.	*Proteobacteria*	*Colletotrichum gloeosporioids*	Kejela et al. (2017)
		Fusarium oxysporum	
Bacillus sp.	*Firmicutes*	*Fusarium oxysporum*	Abdallah et al. (2017)
Paenibacillus ehimensis MA2012	*Firmicutes*	*Colletotrichum gloeosporioides*	Seo et al. (2016)
Pseudoalteromonas piscicida	*Proteobacteria*	*Aspergillus niger*	Paulsen et al. (2016)
		Botrytis cinerea	
Burkholderia cenocepacia VBC7	*Proteobacteria*	*Rhizopus stolonifer*	Ghosh et al. (2015)
Pseudomonas poae VBK1	*Proteobacteria*		

(continued)

Table 18.10 (continued)

Bacteria	Phylum	Antagonistic against	References
Streptomyces vinaceusdrappus S5MW2	*Actinobacteria*	*Rhizoctonia solani*	Yandigeri et al. (2015)
Streptomyces scabrisporus	*Actinobacteria*	*Bipolaris sorokiniana*	Wang et al. (2015)
		Fusarium oxysporum	
		Rhizoctonia solani	
		Phytophthora capsici	
Streptomyces sporovirgulis	*Actinobacteria*	*Alternaria alternata*	Brzezinska et al. (2014)
S. rimosus		*Fusarium solani*	
Brevibacillus laterosporus	*Firmicutes*	*Fusarium equiseti*	Prasanna et al. (2013)
Aeromonas hydrophila SBK1	*Proteobacteria*	*Aspergillus flavus*	Halder et al. (2013)
		Fusarium oxysporum	
Stenotrophomonas maltophilia	*Proteobacteria*	*Fusarium solani*	Suma and Podile (2013)
		F. oxysporum	
		Rhizoctonia solani	
		Alternaria alternata	
Bacillus cereus IO8	*Firmicutes*	*Botrytis cinerea*	Hammami et al. (2013)
Stenotrophomonas maltophilia	*Proteobacteria*	*Fusarium solani*	Jankiewicz et al. (2012)
		F. oxysporum	
		Rhizoctonia solani	
		Alternaria alternata	
Streptomyces roseolus DH	*Actinobacteria*	*Aspergillus* sp.	Jiang et al. (2012)
		Rhizopus chinensis	
		Penicillium sp.	
		Mucor sp.	
Serratia marcescens B4A	*Proteobacteria*	*Rhizoctonia solani*	Zarei et al. (2011)
		Bipolaris sp.	
		Alternaria raphani	
		A. brassicicola	
Serratia sp. CN-01	*Proteobacteria*	*Fusarium oxysporum*	Amin et al. (2011)
Serratia sp. CN-07	*Proteobacteria*		
Pseudomonas sp. CN-05	*Proteobacteria*		
Pseudomonas fluorescens	*Proteobacteria*	*Rhizoctonia solani*	El-Mougy et al. (2011)
		Fusarium solani	
Streptomyces tendae TK-VL_333	*Actinobacteria*	*Aspergillus niger*	Kavitha and Vijayalakshmi (2011)
		Fusarium oxysporum	
Bacillus thuringiensis subsp. *colmeri* 15A3	*Firmicutes*	*Rhizoctonia solani*	Liu et al. (2010)
		Botrytis cinerea	
		Penicillium chrysogenum	
		P. piricola	
		P. glaucum	
		Sclerotinia fuckelian	

(continued)

Table 18.10 (continued)

Bacteria	Phylum	Antagonistic against	References
Bacillus pumilus SG2	*Firmicutes*	*Fusarium graminearum*	Ghasemi et al. (2010)
		Rhizoctonia solani	
		Magnaporthe grisea	
		Sclerotinia sclerotiorum	
		Trichoderma reesei	
		Botrytis cinerea	
		Bipolaris sp.	
Streptomyces sp. DA11	*Actinobacteria*	*Aspergillus niger*	Han et al. (2009)
		Candida albicans	
Rhizobium sp.	*Proteobacteria*	*Aspergillus flavus*	Sridevi and Mallaiah (2008)
		A. niger	
		Curvularia lunata	
		Fusarium udum	
Streptomyces hygroscopicus	*Actinobacteria*	*Colletotrichum gloeosporioides*	Prapagdee et al. (2008)
		Sclerotium rolfsii	
Bacillus cereus YQQ 308	*Firmicutes*	*Fusarium oxysporum*	Chang et al. (2007)
		F. solani	
		Pythium ultimum	
Serratia plymuthica C-1	*Proteobacteria*	*Phytophthora capsici*	Kim et al. (2007)
Chromobacterium sp. C-61	*Proteobacteria*	*Rhizoctonia solani*	
Lysobacter enzymogenes C-3	*Proteobacteria*	*Fusarium oxysporum*	
		F. solani	
Bacillus licheniformis	*Firmicutes*	*Rhizoctonia solani*	Kamil et al. (2007)
B. licheniformis	*Firmicutes*	*Macrophomina phaseolina*	
B. thuringiensis	*Firmicutes*	*Fusarium culmorum*	
Stenotrophomonas maltophilia	*Proteobacteria*	*Pythium* sp.	
		Alternaria alternata	
		Sclerotium rolfsii	
Streptomyces halstedii AJ-7	*Actinobacteria*	*Alternaria alternata*	Joo (2005)
		Botrytis cinerea	
		Fusarium oxysporum	
Enterobacter sp. NRG4	*Proteobacteria*	*Fusarium moniliforme*	Dahiya et al. (2005)
		Aspergillus niger	
		Mucor rouxii	
		Rhizopus nigricans	
Vibrio pacini	*Proteobacteria*	*Mucor racemosus*	Bao-qin et al. (2004)
		Trichoderma viride	
		Zygorhynchus heterognmus	

(continued)

Table 18.10 (continued)

Bacteria	Phylum	Antagonistic against	References
Pseudomonas sp.	*Proteobacteria*	*Macrophomina phaseolina*	Gohel et al. (2004)
Pantoea dispersa	*Proteobacteria*	*Fusarium* sp.	
Enterobacter amnigenu	*Proteobacteria*		
Serratia plymuthica HRO-C48	*Proteobacteria*	*Botrytis cinerea*	Frankowski et al. (2001)
Serratia marcescens strain B2	*Proteobacteria*	*Botrytis cinerea*	Someya et al. (2001)
Alcaligenes xylosoxydans	*Proteobacteria*	*Fusarium* sp.	Vaidya et al. (2001)
		Rhizoctonia bataticola	
Bacillus sp. 739	*Firmicutes*	*Fusarium oxysporum*	Melent'ev et al. (2001)
		F. culmorum	
		Helminthosporium sativum	
Serratia marcescens	*Proteobacteria*	*Sclerotinia minor*	Tarabily et al. (2000)
Streptomyces viridodiasticus	*Actinobacteria*		
Micromonospora carbonacea	*Actinobacteria*		
Paenibacillus sp. 300	*Firmicutes*	*Fusarium oxysporum*	Singh et al. (1999)
Streptomyces sp. 385	*Actinobacteria*		
Bacillus sp. BG-11	*Firmicutes*	*Rhizopus arrhizus*	Bhushan (1998)
		Rhizoctonia solani	
		Sclerotium rolfsii	
		Phytophthora infestans	
		Fusarium oxysporum	
		Phanerochaete chrysosporium	
Serratia marcescens	*Proteobacteria*	*Sclerotium rolfsii*	Ordentlich et al. (1988)

transformation of these chitinous assemblies (Merzendorfer and Zimoch 2003). Thus, addition of chitinolytic enzymes can interrupt in the basic functional progressions similar to ecdysis and redevelopment of peritrophic membrane. Reports suggest chitinase enhanced destruction to the peritrophic membrane of the insect gut (Subbanna et al. 2018). In that way, the creation of a less operative barricade results in appreciable decline in feeding and reduction in the proficiency of digestive procedure, nutritional consumption, and growth. Apart from the straight destruction of peritrophic membrane, chitinases can also perform physical malformations in midgut epithelial cells, like bloating, elongations, and creations of several vacuoles (Terra and Ferreira 2005; Otsu et al. 2003; Gongora et al. 2001; Wiwat et al. 2000).

As the exo-skeletal and other portions of the insects are made up of chitin, prospective chitinolytic bacterial isolates are taking place as a promising biopesticide in the field of improvised biotechnology (Singh et al. 2016). Biocontrol of such insects through potent chitinolytic bacteria is reported so far and can be applied as

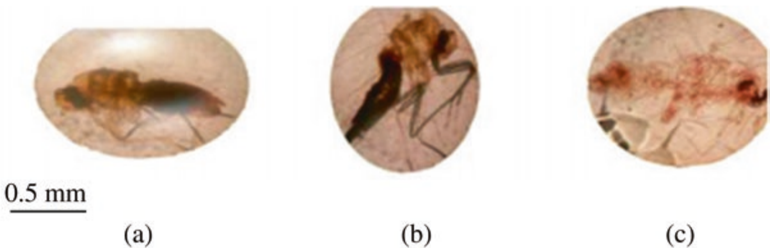

Fig. 18.13 Degradation of whitefly (*Bemisia tabaci*) exoskeleton with *Bacillus cereus* chitinase [(**a**) Control whitefly; (**b**) day 1, treatment with bacterial chitinase; (**c**) day 3, degradation of insect exoskeleton] (Mubarik et al. 2010)

insecticides to control these plant pests (Merzendorfer and Zimoch 2003). According to Aggarwal et al. (2015), a potent chitinase producer, *Serratia marcescens*, demonstrates the highest mortality range of *Spodoptera litura* larvae up to the level 70.8%. Another evidence shows the efficiency of *Bacillus cereus* as a biocontrol agent upon agronomic pest like *Bemisia tabaci* (Mubarik et al. 2010). The potentiality of exoskeleton degradation of the whitefly treated with chitinase isolated from *B. cereus* is given in Fig. 18.13. Keeping the evidences alive, Otsu et al. (2003) exhibit that chitinase-secreting *Alcaligenes paradoxus* KPM-012A was exploited as a biocontrol agent of phytophagous ladybird beetles *Epilachna vigintioctopunctata*. The use of biocontrol agent *Bacillus thuringiensis* H1 has a promising effect on different stages of *Musca domestica* lifecycles (Salama et al. 2016).

Reports regarding the significant plant pest control by the chitinolytic bacteria are reported in such forms like larval developmental control of pests and can be exemplified by *Trichoplusia ni* (Broadway et al. 1998), *Helicoverpa armigera* (Chandrasekaran et al. 2012; Singh et al. 2016), and *Malacosoma neustria* (Danismazoglu et al. 2015) and sucking pests like *Myzus persicae* (Broadway et al. 1998; Rahbe and Febvay 1993), *Bemisia argentifolii*, *Hypothenemus hampei* (Broadway et al. 1998), and *Hypothenemus hampei* (Martínez et al. 2012).

18.4.4 Antagonistic Effect Against Nematodes

Apart from the antifungal and insecticidal fitness, the chitinolytic bacteria also exhibit their nematicidal property against the plant parasites. Nematodes are key agricultural pests of potatoes and in some other crops. Economic crop miscarriage can happen when the nematode population in soil is extraordinarily high. Chemical nematicides are operative but are very toxic to humans and are environmentally hurtful. In search of such alternative, certain bacteria can diminish nematode mobility (Stirling 1984), while other bacteria are on the right path and can produce combinations lethal to plant-parasitic nematodes (Sikora 1991; Spiegel et al. 1991; Oostendorp and Sikora 1990). One such investigation is chitinase-producing soil isolates like *Chromobacterium* sp. UP1 and *Stenotrophomonas maltophilia* MI-12, which inhibited egg hatch of the potato cyst nematode, *Globodera rostochiensis*, up

to 70% as the main constituent of the eggshell of *G. rostochiensis* is chitin (Cronin et al. 1997; Clarke and Hennessy 1976).

Nematode eggs are mainly composed of chitin as the chief structural ingredient. This chitinous facility offers resistance counter to chemical and biological nematicides (Wharton 1980). Chitinases are known to affect egg hatching of many parasitic nematodes like *Meloidogyne hapla* (Mercer et al. 1992), *M. incognita* (Lee and Kim 2015; Nguyen et al. 2007; Jung et al. 2002), *M. javanica* (Spiegel et al. 1991), and *M. arenaria* (Kalaiarasan et al. 2006) by disfiguring and vandalizing the egg shells, leading to either suppression of hatching (Cronin et al. 1997; Lee and Kim 2015) or premature exposure of juveniles which are ineffectual to persist in soil environment (Jung et al. 2002). However, some studies reported discrepancy in susceptibility of eggs and juvenile to chitinases.

In connection with antifungal, insecticidal, and nematicidal properties, there is an upsurge of attention to evolve environment-friendly plant pest-controlling substitutions like chitinase-producing bacteria. This investigation was conducted to travel the unexplored areas of chitinolytic microbes' hub and their possible application as a green pesticide.

18.5 Conclusions

Chitin in the environment is both abundant and prevalent at the same time. Actually, it is the second most abundant biodegradable biopolymer on earth, next to cellulose. Chitin is found in many lifeforms, such as shells (shrimps and crabs), exoskeletons and gut linings of arthropods (crustaceans and insects), and cell walls of several fungi, including some yeasts and structural framework unit of some protista as well as of nematode eggs. The biomolecules that can solubilize that inflexible chitin are known as chitinases. Chitinase can be produced from bacteria, fungi, viruses, plants, and human also. Plant chitinase is produced as a PR protein in response to its defense mechanisms. Bacterial chitinases are recorded from different natural resources like diverse soil and water habitats and shrimp and crab shell waste and also from altered gut systems. Numerous varieties of soil environments are the residence of so many types of chitinolytic bacterial groups. The variants of soil backgrounds are ranging from Antarctic to mangrove, vineyard, agricultural field, and rhizospheric soils of several categories like tea, mango, wheat, maize, rice, and pepper plants. Chitinase-producing bacteria are the resident among the wide range of water bodies from marine to freshwater, hot spring, irrigation well, Lonar lake, shrimp pond, and moat water. These chitinolytic bacteria are the dwellers not only of soil and water but also of shrimp and crab waste dumping area. Interestingly, they are also reported as plant endophytes of agronomic plant parts like root, stem, and leaves. Apart from the rhizospheric soil appearance to endophytic residence, chitinase producers are also present in both the vertebrate and invertebrate gut environments such as fish, bat, insect, and earthworm.

In connection with the abundance of the chitinolytic bacteria in both the endophytic and the endozoic manner, it can be stated that these chitinase-producing

bacteria can deliver metabolic competences, necessary nutrients, and protection against pathogens through enzymatic performances which seem to share evolutionary trends. Many microbial genomes possess different genes encoding chitinolytic enzymes, which have been extensively investigated, but studies regarding the use of microorganisms that utilize insoluble chitin as a carbon source in the area of gut system are sparse. Study of chitinolytic gut microflora is in its infancy; only a few have been studied in adequate detail. As there is versatility within the animal population in terms of population size, habitat, feeding habit, etc., it may be expected that gut microflora can be a gem container consisting of several chitinase producers.

Reported investigations regarding the uses of chitinases and potent chitinolytic microorganisms especially bacteria in the biotechnologically advanced sustainable agriculture are receiving immense attention. From the biocontrol potentiality to biofertilizing ability, these microorganisms approach a new bio-based concept that can reduce the use of chemical fungicides, pesticides, and fertilizers with the assistance of such natural chitinase producers. These chitinolytic bacteria can, therefore, be used as a raw material in biotechnology for environmentally safe and affordable agriculture that leads to human welfare.

18.6 Future Perspectives

- Fungicidal and insecticidal activity of bacterial chitinase may supplement the use of chemical fungicides and insecticides.
- Bioaccumulation of fungicide and insecticide in agronomic crop fields leads to human health risk by biomagnification.
- Inductive plant defense mechanism through the by-products of microbial chitinases like chitooligomers and monomers will secure more pest control potentiality.
- Formulation of microbes as biofertilizers with capabilities like plant growth-promoting traits can create a novel biotechnologically advanced agronomic tool.

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Endophytic Fungi As the Alternate Source of High-Value Plant Secondary Metabolites

19

Sunil K. Deshmukh, Manish K. Gupta, and Sangram K. Lenka

Abstract

Endophytic fungi are associated with plants and reside inside it without causing any harm to the host plants. These fungi are considered as one of the natural sources for getting diverse high-value compounds. Natural compounds which are primarily produced by the host plants including Taxol, camptothecin, podophyllotoxin, vinblastine, vincristine, and huperzine A are also produced by these fungi. Therefore, these endophytic fungi can be considered as an alternate source of high-value natural compounds. The medicinally important plant metabolites isolated from endophytic fungi, their biological properties, and the challenges associated with it are presented in this chapter. Various methods used to optimize the culture conditions, including one strain many compounds (OSMAC), response surface method (RSM), and the genetic tools along with other techniques like cocultivation and epigenetic modification to overcome the problem of attenuation of metabolite synthesis, are also discussed.

Keywords

Attenuation · Cocultivation · Epigenetic modification · Endophytic fungi · High-value plant · Secondary metabolites

19.1 Introduction

Natural products from medicinal plants and microorganisms are the most consistent productive source for the “first-in-class” drugs (Newman and Cragg 2016). Among the natural sources, fungi are getting considerable attention as one of the important

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sources after the discovery of penicillin (Chain et al. 1940) followed by cephalosporins (Brotzu 1948; Abraham and Newton 1961), griseofulvin (Oxford et al. 1939), and statins (Endo 2008; Misra 2016) from various fungal sources. The twenty-first-century antifungal drugs like anidulafungin, Cancidas, and micafungin (Cabello et al. 2001; Morrison 2006; Akihiko 2007; Vicente et al. 2016) are also of fungal origin. Endophytic fungi reside inside the plant without causing any harm to host plants. It is estimated that more than one million species of endophytic fungi coexist with plants based on a ratio of vascular plants to fungal species of 1:4 or 1:5 (Sun and Guo 2012). They are ubiquitous and reside within all known plants, including a broad range of host orders, families, genera, and species across diverse ecosystems. These plants include mosses (Davey and Currah 2006), lichens (Suryanarayanan and Thirunavukkarasu 2017; Suryanarayanan et al. 2005), ferns (Del Olmo-Ruiz and Arnold 2014), grasses (Su et al. 2010; Tanaka et al. 2012), shrubs (Petrini et al. 1982; Naik et al. 2008), tropical dry thorn forest (Murali et al. 2007), and coniferous (Sun et al. 2011; Yoo and Eom 2012) trees. Endophytic fungi belong to mainly the members of the *Ascomycota* or their mitosporic fungi, as well as some taxa of the *Basidiomycota*, *Zygomycota*, and Oomycota (Zheng and Jiang 1995; Sinclair and Cerkauskas 1996; Guo 2001). Endophytic fungi got a lot of attention due to the detection of taxol in *Taxomyces andreanae* by Stierle et al. (1993), a billion-dollar drug industry. After the discovery of taxol, a large number of compounds have been discovered from endophytic fungi, which include camptothecin, podophyllotoxin, vinblastine, vincristine, huperzine A, azadirachtins, rohitukine, piperine, hypericin, berberine, swainsonine, solamargine, withanolide, homoharringtonine, tropane alkaloids, gastrodin, ephedrine hydrochloride, chrysin, sanguinarine, cajanol, and gymnemagenin, to name a few. Metabolites derived from endophytic fungi comprise different classes of compounds such as steroids, xanthones, phenols, isocoumarins, perylene derivatives, quinines, furandiones, terpenoids, depsipeptides, and cytochalasins (Gunatilaka 2006; Deshmukh 2018). These compounds exhibited various biological activities, viz., antibacterial (Deshmukh et al. 2015), antifungal (Deshmukh et al. 2018a), anticancer (Verekar et al. 2014; Deshmukh et al. 2018b, 2019), antiviral (Zhang et al. 2011a), antidiabetic (Mishra et al. 2013), anti-inflammatory (Deshmukh et al. 2009), antioxidant (Tian et al. 2016), volatile antibiotic (Saxena 2016), insecticidal (Kusari et al. 2012), immunosuppressive (Liu et al. 2016a), promoting host growth and resistance to environmental stresses (Cheplick et al. 1989; Ting et al. 2008; Saikkonen et al. 2010; Bilal et al. 2018), and decompose litter (Purahong and Hyde 2011; Sun et al. 2011).

Endophytic fungi are being considered as alternate sources of the bioactive metabolites primarily produced by plants. Utilization of these fungi for the production will maintain consistent market supply of plant natural products with less hassle in cultivation and no adverse environmental effect on production. This technology has the potential not only to conserve the world's diminishing biodiversity but also help in reducing drug cost as it can be produced via fermentation (Suffness 1995). However, so far no natural products of endophytic fungal origin have reached the market due to some challenges associated with their production. The main problem is attenuation of endophytic microorganism and low yield of the desired compound.

This chapter reports the high-value natural products initially isolated from plants but later on reported from endophytic fungi, their biological role, and various methods used for screening and enhancing production of bioactive compounds. The lists of selected high-value plant secondary metabolites from endophytic fungi and their host plants are given in Tables 19.1, 19.2, 19.3 and 19.4, and the structures of the isolated compounds are given in Figs. 19.1 and 19.2.

19.1.1 Taxol

Paclitaxel (Taxol®) is a cyclodecane which was purified from the bark of the Pacific yew tree, *Taxus brevifolia* (Wani et al. 1971), and produced in extremely low quantity in the needles, bark, and roots of yews (*Taxus* sp.). Stierle et al. (1993) discovered *Taxomyces andreanae* from the Pacific yew (*Taxus brevifolia*), the paclitaxel-producing endophytic fungus. This discovery generated interest in the scientific community for the isolation of endophytic fungi producing taxol and other bioactive metabolites, and there have been several reports on taxol producing fungi from yews (Li et al. 2010; Zheng et al. 2010; Hao et al. 2013; Heinig et al. 2013; Kasaei et al. 2017) and other plant species (Bao et al. 2010; Merlin et al. 2012; Gokul et al. 2015; Jayanthi et al. 2015). Paclitaxel and its analogues such as baccatin III and 10-deacetyl-baccatin III have been purified from a large number of endophytic fungi and producers reported after 2012 onward are given in Table 19.1. The hosts of taxane-producing fungi mainly include *Taxus* species (i.e., *T. baccata*, *T. brevifolia*, *T. celebica*, *T. chinensis*, *T. cuspidate*, *T. globosa*, *T. mairei*, *T. media*, *T. sumatrana*, *T. wallachiana*, *T. wallichiana* var. *mairei*, and *Taxus yunnanensis*) belonging to the family Taxaceae, and non-*Taxus* species such as *Hibiscus rosa-sinensis* (Malvaceae), *Podocarpus gracilior*, *Podocarpus* sp. (Podocarpaceae), *Taxodium distichum*, *Taxodium mucronatum.* (Taxodiaceae), *Corylus avellana* (Betulaceae), *Ficus infectoria* (Moraceae), *Justicia gendarussa* (Acanthaceae), *Larix leptolepis* (Pinaceae), *Melochia corchorifolia* (Malvaceae), *Michelia champaca* (Magnoliaceae), *Morinda citrifolia* (Rubiaceae), *Moringa oleifera* (Moringaceae), *Plumeria acutifolia* (Apocynaceae), *Tabebuia pentaphylla* (Bignoniaceae), *Tylophora indica* (Apocynaceae), and mangrove plant *Rhizophora annamalayana* (Rhizophoraceae). This indicates that there is a varied collection of both taxane-synthesizing fungi and their host's plants having a wide-ranging biological diversity (Zhao et al. 2011a; Deshmukh and Verekar 2014; Uzma et al. 2018).

Soliman and Raizada (2013) used cocultivation strategy for yield improvement and observed that 2.7-fold increase in taxol production compared to the control when *Paraconiothyrium* SSM001, a taxol-producing endophyte isolated from *Taxus* x *media*, was cocultured with the *Alternaria* sp., an endophyte associated with *Taxus* (yew) trees. The cultures of *Alternaria* sp. did not show any taxol production alone. Similarly, cultures of *Phomopsis* (one needle isolate) alone did not show any taxol production, while coculturing *Paraconiothyrium* SSM001 with *Phomopsis* led to 3.8-fold increase in fungal taxol. It was also observed when *Paraconiothyrium* SSM001 was cocultured with *Alternaria* sp. isolated from *Taxus* bark and *Phomopsis*

Table 19.1 List of taxol, baccatin III, and 10-deacetyl-baccatin III producing endophytic fungi and their host plants

S/N	Fungus	Plant Source	Compound	References
1	*Gliocladium* sp.	*Taxus baccata*	Taxol	Sreekanth et al. (2011)
2	*Paraconiothyrium* sp.	*Taxus media*	Taxol	Soliman et al. (2011)
3	*Pestalotiopsis* sp. YN6	*Taxus yunnanensis*	Taxol	Zhang et al. (2011c)
4	*Phoma* sp.	*Aloe vera*	Taxol	Rebecca et al. (2011)
5	*Acremonium* sp.	*T. globosa*	Taxol	Soca-Chafre et al. (2011)
6	*Botryosphaeria* sp.	*T. globosa*	Taxol	Soca-Chafre et al. (2011)
7	*Gyromitra* sp.	*T. globosa*	Taxol	Soca-Chafre et al. (2011)
8	*Nigrospora* sp.	*T. globosa*	Taxol	Soca-Chafre et al. (2011)
9	*Lasiodiplodia theobromae*	*Morinda citrifolia*	Taxol	Pandi, et al. (2011)
10	*Didymostilbe* sp. DF110	*Taxus chinensis* var. *mairei*	Taxol	Wang and Tang (2011)
11	*Colletotrichum capsici*	*Capsicum annuum*	Taxol	Kumaran et al. (2011)
12	*Nodulisporium sylviforme*	*Taxus cuspidata*	Taxol	Zhao et al. (2011b)
13	*Phoma betae*	*Ginkgo biloba*	Taxol	Kumaran et al. (2012)
14	*Stemphylium sedicola* SBU-16,	*Taxus baccata*	Taxol, 10-deacetylbaccatin III	Mirjalili et al. (2012)
15	*Pestalotiopsis pauciseta* VM1	*Tabebuia pentaphylla*	Taxol	Vennila, et al. (2012)
16	*Chaetomium* sp.	*Michelia champaca*	Taxol	Rebecca et al. (2012)
17	*Aspergillus carbonarius* BJ-11	*Taxus chinensis*	Taxol	Li et al. (2012a)
18	*Hypocrea* sp. Z58	*Taxus x media*	Taxol	Miao et al. (2012)
19	*Fusarium solani* LCPANCF01	*Tylophora indica*	Taxol	Merlin et al. (2012)
20	*Fusarium oxysporum*	*Rhizophora annamalayana*	Taxol	Elavarasi et al. (2012)
21	*Alternaria* sp.	*Taxus mairei*	Taxol	Zhang et al. (2012)
22	*Colletotrichum gloeosporioides* TA67	*Taxus x media*	Taxol	Xiong et al. (2013)
23	*Cladosporium tenuissimum* H-21	*Taxus chinensis*	Taxol	Ding et al. (2013a)
24	*Guignardia mangiferae* HAA11	*Taxus x media*	Taxol	Xiong et al. (2013)
25	*Fusarium proliferatum* HBA29	*Taxus x media*	Taxol	Xiong et al. (2013)

26	*Fusarium solani*	*Taxus celebica*	Taxol, baccatin III	Chakravarthi et al. (2013)
27	*Fusarium redolens*	*Taxus baccata* subsp. *wallichiana*	Taxol	Garyali et al. (2013, 2014a, b)
28	*Penicillium aurantiogriseum* NRRL 62431	*Corylus avellana*	Taxol	Yang et al. (2014a)
29	*Nodulisporium sylviforme* HDF-68	*Taxus cuspidata*	Taxol	Zhao et al. (2014)
30	*Aspergillus* sp.	*Taxus chinensis*	Taxol	Wang et al. (2014a)
31	*Botryosphaeria* sp. J11	*Taxus chinensis* var. *mairei*	Taxol	Liu et al. (2014)
32	*Cladosporium cladosporioides* UH-10	*Taxus* sp.	Taxol	Gohar et al. (2015)
33	*Cladosporium oxysporum*	*Moringa oleifera*	Taxol	Gokul et al. (2015)
34	*Nodulisporium sylviforme* HDFS4-26	*Taxus cuspidata*	Taxol	Wang et al. (2015)
35	*Phomopsis* sp. MHZ-32	*Taxus x media*	Taxol	Xi et al. (2015)
36	*Phomopsis longicolla*	*Mesua ferrea*	Taxol	Jayanthi et al. (2015)
37	*Trichoderma* sp. IRB54	*Taxus wallichiana*	10-deacetyl baccatin III	Li et al. (2015)
38	*Aspergillus fumigatus* TMS-26	*Taxus media*	Taxol	Gou et al. (2015)
39	*Phoma* sp.	*Calotropis gigantea*	Taxol	Hemamalini et al. (2015)
40	*Paraconiothyrium* SSM001	*Taxus baccata*	Taxol	Soliman et al. (2015)
41	*Paraconiothyrium variabile*	*Taxus baccata*	Taxol	Somjaipeng et al. (2016)
42	*Penicillium aurantiogriseum*	*Corylus avellana*	Taxol	Liu et al. (2016b)
43	*Pestalotiopsis stellate*	*Ficus infectoria*	Taxol	Doss et al. (2016)
44	*Epicoccum nigrum*	*Taxus baccata*	Taxol	Somjaipeng et al. (2016)
45	*Grammothele lineata*	*Corchorus olitorius* acc. (2015)	Taxol	Das et al. (2017)
46	*Cladosporium* sp.	*Taxus baccata*	Taxol	Kasaei et al. (2017)
47	*Phoma medicaginis*	*Taxus wallichiana* var. *mairei*	Taxol	Zaiyou et al. (2017)

(continued)

Table 19.1 (continued)

S/N	Fungus	Plant Source	Compound	References
48	*Aspergillus niger* subsp. *taxi* HD86-9	*Taxus cuspidata*	Taxol, baccatin III, and 10-deacetyl-baccatin III	Li et al. (2017)
49	*Aspergillus fumigatus* TMS-26	*Taxus media*	Taxol	Fang et al. (2017)
50	*Aspergillus aculeatinus* Tax-6	*Taxus chinensis* var. *mairei*	Taxol	Qiao et al. (2017)
51	*Paraconiothyrium* SSM001	*Taxus media*	Taxol	Soliman et al. (2017)
52	*Pestalotiopsis microspora*	*Taxodium mucronatum*	7-epi-10-deacetyltaxol	Subban et al. (2017)
53	*Cladosporium cladosporioides* MD2	*Taxus media*	Taxol, 10-deacetylbaccatin III	Zhang et al. (2009, 2011b) and Miao et al. (2018)
54	*Aspergillus terreus* EFB108, EFB59, EFB14	*Podocarpus gracilior*	Taxol	El-Sayed et al. (2018)

#Refer to Zhao et al. (2011a) and Deshmukh and Verekar (2014) for details of the taxol, baccatin III, and 10-deacetyl-baccatin III producing endophytic fungi and their host plants reported before 2010

Table 19.2 Camptothecin, 9-methoxycamptothecin, and 10-hydroxycamptothecin producing endophytic fungi and their host plants

S/N	Fungus	Plant source	Compound	References
1.	*Phomopsis* sp. B29	*Camptotheca acuminata*	Camptothecin	Wang and Chen (2011)
	Penicillium sp.	*Camptotheca acuminata*	Camptothecin	Li et al. (2011)
2.	*Fomitopsis* sp. *Alternaria alternata*, *Phomposis* sp.	*Miquelia dentata*	Camptothecin, 9-methoxycamptothecin, 10-hydroxycamptothecin	Shweta et al. (2013)
3.	*Trichoderma atroviridae*	*Camptotheca acuminata*	Camptothecin	Pu et al. (2013)
4.	*Botryosphaeria dothidea*	*Camptotheca acuminata*	9-Methoxycamptothecin	Ding et al. (2013b)
5.	*Fusarium nematophilum*	*Camptotheca cuminata*	Camptothecin	Su et al. (2014)
6.	*Alternaria alternata*	*Camptotheca cuminata*	Camptothecin	Su et al. (2014)
7.	*Phomopsis vaccinii*	*Camptotheca cuminata*	Camptothecin	Su et al. (2014)
8.	*Colletotrichum gloeosporioides*	*Camptotheca cuminata*	10-Hydoxycamptothecin	Su et al. (2014)
9.	*Fusarium oxysporum* (NCIM 1383)	*Nothapodytes nimmoniana*	Camptothecin	Bhalkar et al. (2015, 2016a)
10.	*Trichoderma* GZU-BCEC-GX8	*Nothapodytes pittosporoides*	Camptothecin	Lei et al. (2016)
11.	*Fusarium solani*	*Camptotheca acuminata*	40μg/g	Ran et al. (2017)

#Refer to Zhao et al. (2011a) and Deshmukh and Verekar (2014) for details of the camptothecin, 9-methoxycamptothecin, and 10-hydroxycamptothecin producing endophytic fungi and their host plants reported before 2010

Table 19.3 List of podophyllotoxin producing endophytic fungi and their host plants

S/N	Fungus	Plant source	Compound	References
1.	*Alternaria neesex* Ty 18	*Sinopodophyllum hexandrum*	Podophyllotoxin	Cao et al. (2007)
2.	*Alternaria* sp.	*Podophyllum hexandrum*	Podophyllotoxin	Chandra (2012)
3.	*Alternaria* sp.	*Sabina vulgaris*	Podophyllotoxin	Zhao et al. (2010)
4.	*Alternaria* sp.	*Podophyllum hexandrum*	Podophyllotoxin	Zhao et al. (2010)
5.	*Monilia* sp.	*Dysosma veitchii*	Podophyllotoxin	Zhao et al. (2010)
6.	*Penicillium* sp.	*Podophyllum hexandrum*	Podophyllotoxin	Zhao et al. (2010)
7.	*Penicillium* sp.	*Diphylleia sinensis*	Podophyllotoxin	Zhao et al. (2010)
8.	*Fusarium solani*	*Podophyllum hexandrum*	Podophyllotoxin	Nadeem et al. (2012)
9.	*Mucor fragilis* TW5	*Sinopodophyllum hexandrum*	Podophyllotoxin	Huang et al. (2014)
10.	*Alternaria tenuissima*	*Sinopodophyllum emodi*	Podophyllotoxin	Liang et al. (2016)
11.	*Chaetomium globosum* *Pseudallescheria* sp.	*Sinopodophyllum hexandrum*	Podophyllotoxin	Wang et al. (2017)
12.	*Fusarium* sp. (WB5121 and WB5122)	*Dysosma versipellis*	Podophyllotoxin	Tan et al. (2018)

Table 19.4 List of huperzine A producing endophytic fungi and their host plants

Sr. No.	Fungus	Plant source	Compound	References
1	*Acremonium* sp. 2F09P03B	*Huperzia serrata*	Hup A	Li et al. (2007) and Su et al. (2011)
2	*Blastomyces* sp. HA15	*Phlegmariurus cryptomerianus*	Huperzine A	Ju et al. (2009)
3	*Botrytis* sp. HA23	*Phlegmariurus cryptomerianus*	Huperzine A	Ju et al. (2009)
4	*Penicillium rugulosum* g5	*Huperzia serrata*	Huperzine A	Chen et al. (2008)
5	*Acremonium endophytium*	*Huperzia serrata*	Huperzine A	Li et al. (2008)
6	*Penicillium chrysogenum* SHB	*Lycopodium serratum*	Huperzine A	Zhou et al. (2009)
7	*Penicillium chrysogenum* TCM-01		Huperzine A	Hong et al. (2010)
8	*Shiraia* sp. Slf14	*Huperzia serrata*	Huperzine A	Zhu et al. (2010), Yang et al. (2016) and Peng et al. (2016)
9	*Xylarialerna* SY-02	*Huperzia serrata*	Huperzine A	Su et al. (2011)
10	*Aspergillus flavus* LF40	*Huperzia serrata.*	Huperzine A	Wang et al. (2011a)
11	*Cladosporium cladosporioides* LF70	*Huperzia serrata.*	Huperzine A	Zhang et al. (2011d)
12	*Shiraia* sp. Slf14	*Huperzia serrata*	Huperzine A, Hypocrellin A	Yang et al. (2014b)
13	*Podospora species* S29	*Huperzia serrate*	Huperzine A	Dong et al. (2014)
14	*Trichoderma* sp.L44	*Huperzia serrata*	Huperzine A	Dong et al. (2014)
15	Paecilomyces tenuis YS-13	*Huperziaceae*	Huperzine A	Su and Yang (2015)
16	*Ceriporia lacerata* MY183	*Phlegmariurus phlegmaria*	Huperzine A	Zhang et al. (2015a)
17	*Hypoxylon investiens* MY311	*Phlegmariurus phlegmaria*	Huperzine A	Zhang et al. (2015a)
18	*Colletotrichum gloeosporioides* ES026	*Huperzia serrata*	Huperzine A	Zhao et al. (2013a) and Zhang et al. (2015b)
19	*Colletotrichum gloesporioides*	*Huperzia serrata*	Huperzine A Huperzine B	Hu et al. (2018)
20	*Paraboeremia* sp. Lsl3KI076	*Lycopodium serratum* var. *longipetiolatum*	12-epi lycopodine, lycopodine, and huperzine A	Ishiuchi et al. (2018)

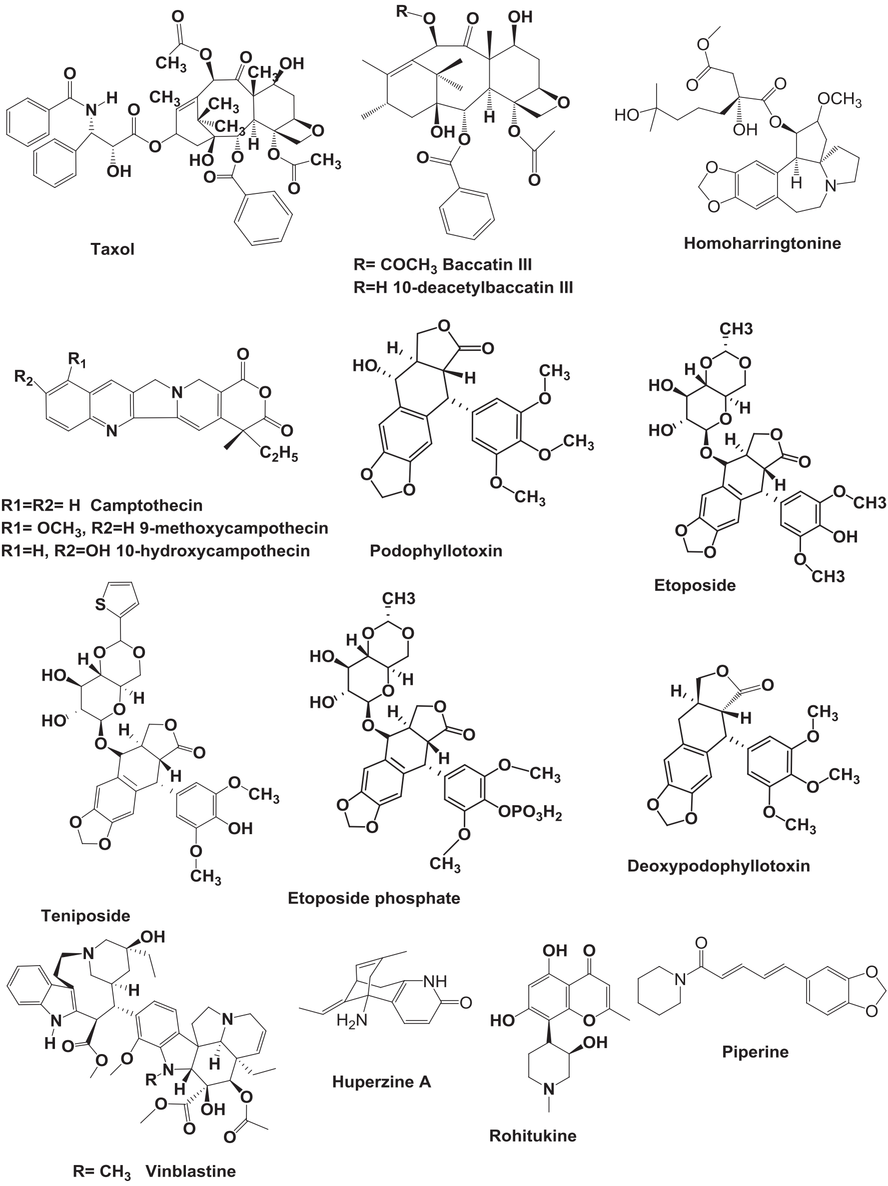

Fig. 19.1 Structures of metabolites isolated from endophytic fungi

sp. isolated from *Taxus* needles caused a 7.8-fold increase in fungal taxol production (Soliman and Raizada 2013).

It is important to note here that 19 enzymatic conversion steps (Croteau et al. 2006) are involved following the formation of geranylgeranyl diphosphate (GGPP).

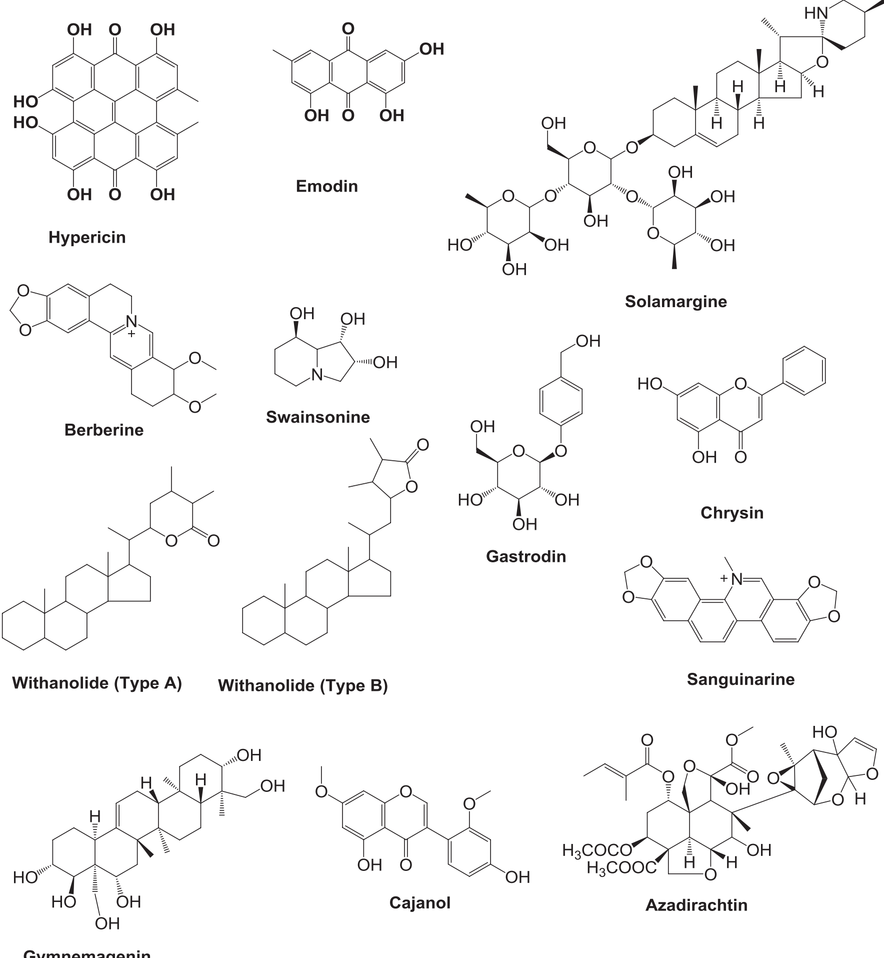

Fig. 19.2 Structures of metabolites isolated from endophytic fungi

Cyclization of GGPP to taxadiene follows eight oxidations, five acyl/aroyl transferases, one epoxidation, one aminomutase, two CoA esterifications, and an N-benzoylation step to form paclitaxel. Unknown steps include key reactions that lead to the formation of the oxetane ring ultimately producing the intermediate compound 10-deacetyl-2-debenzoylbaccatin III. The gene(s) involved in oxetane ring formation still remains unidentified in taxadiene biosynthesis. Therefore, it is important to identify the signature genes in endophytes that could catalyze in the uncharacterized steps of the paclitaxel biosynthetic pathway (e.g., hydroxylation reactions and oxetane ring formation) for independent taxadiene biosynthesis by the fungus. Further, the low yield of the metabolite (taxanes) in endophytes could be attributed to the detoxifying endophytic genes encoding enzymes that either divert

taxanes into less toxic derivatives of taxol biosynthetic pathway or prevent higher product accumulation or due to the action of endophyte-encoded enzymes responsible for degradation of paclitaxel or other taxanes. Study on these novel genes would be significant to define the precise activity of the encoded proteins. The practice of coculturing different endophytes to produce more paclitaxel is possibly achieved due to the movement of substrates between multiple endophytic cellular compartments implicated in efficient taxane synthesis, transport, and accumulation. This, in turn, might reduce the cytotoxicity to endophytes due to taxane accumulation and adequate supply of precursors ensures completion of multiple downstream steps of the biosynthesis of taxanes. Taxol excretion in culture media is another aspect to be investigated. Taxol transport facilitates an active biosynthesis of taxol possibly by reducing the feedback inhibition processes or reducing cytotoxicity in the cytoplasm in the presence of taxol in the desired endophytes.

The comparative transcriptomics of cultured taxol-producing endophyte cells exhibiting metabolic and biological diversity will further help us in understanding endophytic taxol biosynthesis. This analysis will provide a valuable insight for better understanding of the global regulation of paclitaxel biosynthesis. The transcripts are reported in taxol-producing and nonproducing state of cultured cells; this will facilitate identifying rate-limiting steps of this pathway in the fungus. Along with paclitaxel biosynthetic pathway, the phenylpropanoid pathway also has to be studied; this was supported by the accumulation of phenylpropanoid by-products in the cultures. The molecular phenotype taxol producing endophyte culture cells is yet to be elucidated. As taxol biosynthetic genes are co-regulated in response to methyl jasmonate (MeJA) in plants (Lenka et al. 2012), their response in fungus needs to be studied in more details. An increase in the phenylpropanoid pathway upon MeJA or any other elicitation will help in enhancing the overall accumulation of different taxanes in the cultures and engineering strategies for sustainable taxol production in endophytes.

Taxol possesses anticancer activity against a variety of cancer including breast, ovarian, lung, esophageal, bladder, prostate, melanoma, as well as other types of solid tumors. Taxol preferentially binds to microtubules and stabilizes the microtubule assembly against tubulin depolymerization. It promotes the microtubule polymerization by shifting the tubulin dimer-polymer equilibrium facilitating polymer assembly. Interestingly, taxol binding site in microtubules is different from that of other anti-microtubule agents such as colchicine, vinca alkaloids, and podophyllotoxin which promote disassembly of microtubules (Foa et al. 1994). Following exposure of HeLa cells and fibroblasts to taxol (0.25 or 10 μM/dm3), there was growth arrest in the G2 and M phases of the cell cycle (Nikolic et al. 2011).

19.1.2 Homoharringtonine

Homoharringtonine (omacetaxine mepesuccinate) is a cytotoxic alkaloid originally isolated from the evergreen tree, *Cephalotaxus hainanensis.* It is a translation elongation inhibitor and binds to the 80S ribosome in eukaryotic cells and inhibits

protein synthesis by interfering with chain elongation. Homoharringtonine blocks progression of leukemic cells from G1 phase into S phase and from G2 phase into M phase. Hu et al. (2016) purified homoharringtonine from *Alternaria tenuissima* CH1307 an endophyic fungus associated with the *Cephalotaxus hainanensis* (Hu et al. 2016).

Homoharringtonine is an alkaloid with a cephalotaxine nucleus. It possesses strong antiproliferative activity. Homoharringtonine is a protein translation inhibitor, an agent, and inhibits the elongation step of protein synthesis. It is very effective in cancer patients who are resistant to multiple tyrosine kinase inhibitors (sorafenib and imatinib target). Therefore, homoharringtonine was approved by the FDA in 2012 (trade name Synribo®) for its use in the treatment of chronic myeloid leukemia in patients with resistance and/or intolerance to two or more tyrosine kinase inhibitors and is the only approved drug for the treatment of chronic myeloid leukemia (Seca and Pinto 2018).

19.1.3 Camptothecin

Camptothecin, isoquinoline alkaloid, was first discovered in the Chinese deciduous tree, *Camptotheca acuminata* (Nyssaceae) (Wall et al. 1966), later on reported from *Nothapodytes nimmoniana* (Govindachari and Viswanathan 1972; Fulzele et al. 2001; Suhas et al. 2007). *Nothapodytes nimmoniana*, was formerly known as *Nothapodytes foetida* Sleumer and also as *Mappia foetida* Meirs. Subsequently, camptothecin was purified from several endophytes including *Entrophosphora infrequens* residing inside the bark of *Nothapodytes foetida* (Puri et al. 2005) followed by from *Neurospora crassa*, a seed endophyte of *Camptotheca acuminata* (Rehman et al. 2008), from *Fusarium solani* associated with *Camptotheca acuminata* (Kusari et al. 2009a), from *Nodulisporium* sp. residing inside *Nothapodytes foetida* (Rehman et al. 2009), *Botryosphaeria parva residing* inside *Nothapodytes nimmoniana* (Icacinaceae) (Gurudatt et al. 2010), *Fusarium solani* strains MTCC9667 and MTCC9668, the endophytes associated with *Apodytes dimidiate* (Shweta et al. 2010), *Penicillium* sp. isolated from *Camptotheca acuminata* (Li et al. 2011), *Trichoderma atroviridae* isolated from *Camptotheca acuminata* (Pu et al. 2013), *Fusarium nematophilum*, *Alternaria alternata*, *Phomopsis vaccinia* isolated from *Camptotheca cuminata* (Su et al. 2014), *Fusarium oxysporum* (NCIM 1383) isolated from *Nothapodytes nimmoniana* (Bhalkar et al. 2015, 2016a), *Trichoderma* sp. GZU-BCECGX8 isolated from *Nothapodytes pittosporoides* (Lei et al. 2016), and *Fusarium solani* isolated from *Camptotheca acuminata* (Ran et al. 2017) [Table 19.2].

Two analogues of camptothecin, 9-methoxycamptothecin and 10-hydroxycamptothecin, were extracted from *Fusarium solani*, associated with *Camptotheca acuminata* (Kusari et al. 2009a). 10-Hydroxycamptothecin was also purified from an unidentified endophytic fungal strain XK001 associated with *Camptotheca acuminata* (Min and Wang 2009) and from *Xylaria* sp. M20

endophytic fungus isolated from *Camptotheca acuminata* (Liu et al. 2010). 9-Methoxycamptothecin and 10-hydroxycamptothecin were also purified from *Fusarium solani* associated with *Apodytes dimidiate* collected from the Western Ghats, India (Shweta et al. 2010). Later on, 9-methoxycamptothecin was also purified from *Botryosphaeria dothidea* isolated from *Camptotheca acuminata* (Ding et al. 2013b); camptothecin, 9-methoxycamptothecin, and 10-hydroxycamptothecin were purified from *Fomitopsis* sp., *Alternaria alternata*, and *Phomposis* sp. isolated from *Miquelia dentata* (Shweta et al. 2013); and 10-hydroxycamptothecin was purified from *Colletotrichum gloeosporioides* isolated from *Camptotheca cuminata* (Su et al. 2014). Up to 15.5-fold enhanced production of camptothecine was observed when ethanol (up to 5% vol./vol.) was added to the suspension culture of the camptothecin producing endophytic fungal culture *Fusarium solani*, known to produce camptothecin obtained from *Catharanthus roseus.* Enhanced production of camptothecin was observed when ethanol (up to 5% vol./vol.) was added to the suspension culture of the endophyte *Fusarium solani*, known to produce camptothecine obtained from *Catharanthus roseus*; it was increased up to 15.5-fold.

It may be due to dual role of ethanol, presumably as an elicitor and also as a carbon/energy source, leading to enhanced biomass and camptothecin production (Venugopalan and Srivastava 2015). Mixed fermentation of two endophytic fungi, viz., *Colletotrichum fructicola* SUK1 (F1) and *Corynespora cassiicola* SUK2 (F2), isolated from *Nothapodytes nimmoniana* yielded up to 146 mg/L of camptothecine while individually yielded camptothecine up to 33 mg/L and 69 1.1 mg/L, respectively (Bhalkar et al. 2016b). A strain of *Penicillium* sp. was found to be capable of transforming camptothecin into 10-hydroxycamptothecin; the convention rate was 7.37% (Wang et al. 2014b).

Various methods have been tried to restore the camptothecine. In some cases, it was observed that camptothecine production is restored when the attenuated endophytic fungi were isolated from camptothecine-producing plants, *Nothapodytes nimmoniana* and *Miquelia dentate*. In another method, attenuated endophytic fungus that was labeled with green fluorescent protein (GFP) was used to track the fungus through the process of inoculation and its re-isolation. There was significant enhancement of production observed compared to attenuated fungus. It was observed that attenuated endophytic fungi that traversed through their host tissue or plants capable of synthesizing camptothecine produced significantly higher camptothecine as compared to the attenuated fungi (Vasanthakumari et al. 2015). In another approach, enhanced production of camptothecine was observed in comparison to attenuated fungus cultured in the presence of a DNA methyltransferase inhibitor 5-azacytidine. This is mainly due to reversal of eliciting some signals from plant tissue, most likely that which prevents the methylation or silencing of the genes responsible for camptothecine biosynthesis (Vasanthakumari et al. 2015). There was 10.6-fold enhancement of camptothecin production in comparison to that in control (2.8 μg/L), which was observed when ethanolic extract of a non-camptothecin-producing plant *Catharanthus roseus* was added in the suspension culture of camptothecin producing *Fusarium solani* strain. The enhanced production was observed in camptothecin production (up to 15.5-fold). It was also observed

that there was maximum enhancement in camptothecin production (up to 15.5-fold) as compared to that in control when pure ethanol (up to 5% $^{v}/_{v}$) was added in the suspension culture of *F. solani*. It was concluded that ethanol acts as an elicitor and also as a carbon/energy source and enhances biomass and camptothecine production (Venugopalan and Srivastava 2015).

Salicylic acid (SA) and methyl jasmonate (MeJA), two known activators of fungal secondary metabolism, two camptothecine biosynthetic precursors tryptamine and secologanin, and four metal ions Ca^{2+}, Cu^{2+}, Li^{+}, and Mn^{2+} were added to the culture for their effects on camptothecine production in the second-generation strains of *Trichoderma atroviride* LY357. With the addition of MeJA and SA, there was 3.4- and 2.2-fold increase, respectively, of camptothecine yield. There was slight increase of camptothecine yield by the addition of Ca^{2+}, Cu^{2+}, and Mn^{2+}, while a slight decrease was observed by the addition of Li^{+}. The slight increase in the yield of camptothecine by feeding with tryptamine, as well as a marginal decrease, was observed by feeding with secologanin (Pu et al. 2013).

The yield of 10-hydroxycamptothecin in submerged culture of *Xylaria* sp. M20 was enhanced by several chemicals including both inorganic ions and organic compounds ($Ce(NO_3)_3$. $6H_2O$, $Cr(NO_3)_3.9H_2O$, $La(NO_3)_3.6H_2O$, indole, tryptophan, salicylic acid (SA), $CuSO_4.5H_2O$, $FeCl_2.H_2O$, $MnSO_4.H_2O$, $CaCl_2$, $NaSeO_3$, and $LiCl.H_2O$). There is an increase in the production of 10-hydroxycamptothecin up to 5.4 mg/L after addition of salicylic acid, which promoted production at 0.1 mM to the suspension culture (Liu et al. 2010)

The effect of different nutrient combinations and precursors (tryptophan, tryptamine, geraniol, citral, mevalonic acid, and leucine) was observed on the accumulation of camptothecine by endophytic fungus *Entrophospora infrequens*. The precursors were added in suspension culture either solitarily or in mixture (tryptophan and geraniol, tryptophan and citral, tryptophan and mevalonic acid, tryptophan and leucine). In the Sabouraud medium, the highest camptothecine content was attained in the range of 503 ± 25μg/100g dry cell mass. Increase of camptothecine content in the medium by 2.5-folds was observed by the presence of tryptophan and leucine, whereas the production with tryptophan was also significantly different from other treatments (Amna et al. 2012).

Camptothecin displays good anticancer efficacy against a wide range of tumors. Camptothecine acts by inhibiting human DNA topoisomerase I (topo-I). It preferentially inhibits topo-I by hindering the rejoining step of the religation reaction of topo-I and led to the accumulation of a covalent reaction intermediate known as topo-I cleavable complex. It was observed that the camptothecine induces the S-phase-specific killing through potentially lethal collisions between advancing replication forks and topo-I cleavable complexes. Camptothecine also interacts with DNA by forming topo-I DNA complexes, which contribute to CPT-mediated cytotoxicity (Liu et al. 2000).

A comparative study determining the cytotoxic effect of camptothecine on HEp-2 cells (human epithelial pharyngeal carcinoma tissue cells) and MRC-5 cells (normal cells) showed that camptothecine inhibited the proliferation of HEp-2 cells in a dose-dependent manner with an IC_{50} of 0.05±0.022 μg/ml, while the inhibitory

effect on MRC-5 cells was only 45% at the camptothecine concentration of 0.6 μg/ml (Sarzaeem et al. 2013).

19.1.4 Podophyllotoxin

Podophyllotoxin, an aryltetralin lignin, was first purified from *Podophyllum emodi* (Leiter et al. 1950) and currently in use to treat genital warts. Its derivatives, etoposide, teniposide, and etoposide phosphate, the topoisomerase I inhibitors, serve as anticancer drugs (Puri et al. 2006; Eyberger et al. 2006). Later on, podophyllotoxin was purified from *Trametes hirsute*, an endophytic fungus isolated from the dried rhizomes of *Podophyllum hexandrum* (Puri et al. 2006). It was also extracted from two different strains of *Phialocephala fortinii* associated with the rhizomes of *Podophyllum peltatum.* The yield of podophyllotoxin was in the range of 0.5–189 μg/L in 4 weeks of culture (Eyberger et al. 2006). It was isolated reportedly from *Fusarium oxysporum* associated with *Juniperus recurva* (Kour et al. 2008). Podophyllotoxin was also obtained from an endophytic fungus *Penicillium implicatum* SJ21 isolated from *Diphylleia sinensis*, which grew in Zhongdian County, Yunnan province (Zeng et al. 2004). *Fusarium solani* isolated from the roots of *Podophyllum hexandrum* (Nadeem et al. 2012) and *Alternaria tenuissima* isolated from *Sinopodophyllum emodi* (Liang et al. 2016) also produced podophyllotoxin. *Aspergillus fumigates*, an endophytic fungus associated with *Juniperus communis*, was the source of anticancer pro-drug deoxypodophyllotoxin (Kusari et al. 2009b). More recently, podophyllotoxin was isolated from two strains of *Fusarium* sp. (WB5121 and WB5122) associated with *Dysosma versipellis*, a rare and vulnerable perennial herb that is endemic to China. The highest yield of 277 μg/g was observed in *Fusarium* sp. (WB5121) (Tan et al. 2018). Endophytic fungi as a source of podophyllotoxin and their host plants are given in Table 19.3.

It was observed that the addition of 1 g/L *Piriformospora indica* for 24 h induces maximum increments of 3.4 times (570.3 mg/L) and 4.9 times (65.3 mg/L) in podophyllotoxin and 6-methoxypodophyllotoxin accumulation, respectively, in comparison with control cultures (Baldi et al. 2010). Similarly, improvement of podophyllotoxin (PT) and 6-methoxypodophyllotoxin (6-MPT) production by 3.76 times (628.9 mg/L) and 8.74 times (116.8 mg/L) by addition of 2.5 g/L *Sebacina vermifera* for 24 h in cocultivation experiments was observed (Baldi et al. 2010).

Podophyllotoxin is a potential antitumor agent active against Wilms, lung cancer, lymphomas, and genital tumors. It is a potent inhibitor of microtubule assembly. The mechanism of action of podophyllotoxin includes its binding with tubulin which destabilizes microtubules, thus preventing cell division. Interestingly, etoposide, a podophyllotoxin derivative and anticancer drug, caused cell growth inhibition by inhibiting DNA topoisomerase II, which causes double-strand breaks in DNA; etoposide does not, however, inhibit tubulin polymerization. Likewise, podophyllotoxin has no inhibitory activity against DNA topoisomerase II (Gordaliza et al. 2000; Damayanthi and Lown 1998).

19.1.5 Vinca Alkaloids

Vinblastine and vincristine, two vinca alkaloids, were purified from *Catharanthus roseus*, the Madagascar periwinkle (Gueritte and Fahy 2005). Later on, Guo et al. (1998) isolated vinblastine from *Alternaria* sp., an endophytic fungus associated with *Catharanthus roseus*. Afterward, *Fusarium oxysporum* and an unidentified vincristine-producing endophytic fungus associated with *C. roseus* were the source of vincristine (Zhang et al. 2000; Yang et al. 2004). Both the vinca alkaloids were also reported from *Fusarium oxysporum* and *Talaromyces radicus*-CrP20 isolated from *Catharanthus roseus* (Kumar et al. 2013; Palem et al. 2015). More recently, vinblastine has been extracted from *Nigrospora sphaerica* residing inside *Catharanthus roseus* (yield 0.868 μg/ml) (Ayob et al. 2017). Kumar and Ahmad (2013) reported biotransformation of vinblastine to vincristine by the endophytic fungus *Fusarium oxysporum* isolated from *Catharanthus roseus*.

Vincristine and vinblastine are important anticancer agents known to suppress microtubule dynamics and assembly, resulting in the arrest of cell division at metaphase. They exert their antimitotic action by inhibiting microtubule assembly and induce tubulin self-association into coiled spiral aggregates (Lobert et al. 1996). Vincristine and vinblastine are used in the treatment of acute leukemia, rhabdomyosarcoma, neuroblastoma, Wilm's tumor, Hodgkin's disease, and several nonmalignant hematologic disorders (Sri 2016).

19.1.6 Huperzine A

Huperzine A, a Lycopodium alkaloid, was isolated from the Chinese traditional medicinal plant *Huperzia serrate*. It is a highly specific, reversible inhibitor of acetylcholinesterase (AChE) with low toxicity, improves many cognitive functional defects, and therapeutically treats myasthemia gravis and early-middle Alzheimer's disease (AD). HupA has been marketed in China as a new drug for Alzheimer's disease (AD) treatment and is currently used in the United States as a supplement for preventing further memory degeneration (Zangara 2003). It was also purified from the *Acremonium* sp. (2F09P03B), an endophyte associated with *Huperzia serrata* (Li et al. 2007; Su et al. 2011). The endophytic fungus *Penicillium rugulosum* g5, *Acremonium endophytium*, *Xylarialerna* SY-02, *Aspergillus flavus* LF40, *Cladosporium cladosporioides* LF70, *Shiraia* sp. Slf14, *Podospora* species S29, *Trichoderma* sp.L44, *Paecilomyces tenuis* YS-13, *Shiraia* sp. Slf14 *Penicillium chrysogenum* TCM-01, and *Colletotrichum gloeosporioides* ES026 isolated from *Huperzia serrata* were the source of huperzine A (Chen et al. 2008; Li et al. 2008; Su et al. 2011; Wang et al. 2011a, b; Zhang et al. 2011d; Zhu et al. 2010; Yang et al. 2014b, 2016; Peng et al. 2016; Dong et al. 2014; Su and Yang 2015; Hong et al. 2010; Zhang et al. 2017; Hu et al. 2018). Huperzine A was also purified from endophytic fungus *Blastomyces* sp. (HA15) and *Botrytis* sp. (HA23), *Ceriporia lacerata* MY183, and *Hypoxylon investiens* MY311 isolated from *Phlegmariurus cryptomerianus* (Ju et al. 2009; Zhang et al. 2015a). 12-epi lycopodine, lycopodine, and

huperzine A were also purified from endophytic fungus *Penicillium chrysogenum* SHB, *Paraboeremia* sp. Lsl3KI076 isolated from *Lycopodium serratum* (Zhou et al. 2009; Ishiuchi et al. 2018) (Table 19.4). In case of *C. gloeosporioides* ES026, 28.58% increase in the yield was observed at the optimized temperature, pH, agitation rate, and fermentation period and carbon source. Additionally, it was also observed that small molecule like ethanol can significantly increase the yield of HupA (51.89% increment). The addition of ethanol increases the yield of HupA by 51.89% as compared to the parent strain (Zhao et al 2013a).

The huperzine A possesses reversible acetylcholinesterase (AChE) inhibitory activity and is a potential drug candidate in the treatment of Alzheimer's disease (AD). Zhu et al. (2010) have shown that the huperzine A causes concentration-dependent inhibition of AChE (Zhu et al. 2010). In a phase IV clinical trials in China, huperzine A showed significant improvement in memory shortages in aged people and in patients with AD or vascular dementia, with minimal peripheral cholinergic side effects (Orhan et al. 2011). Patients with AD show cognitive and task switching deficits in contrast to healthy individuals. Gul et al. (2018) have demonstrated that huperzine A treatment significantly improves the cognition and task switching abilities in patients with AD (Gul et al. 2018). HupA acts through α7nAChRs and α4β2nAChRs and produces potent anti-inflammatory response by decreasing IL-1β, TNF-α protein expression, and suppressing transcriptional activation of NF-κB signaling (Damar et al. 2016).

19.1.7 Rohitukine

Rohitukine, a natural occurring chromone alkaloid, was first time extracted from *Amoora rohituka* (family Meliaceae) (Harmon et al. 1979) and later on from *Dysoxylum binectariferum* (Naik et al. 1988; Safia et al. 2015). Rohitukine possesses diverse biological activities including anti-inflammatory, immunomodulatory, anti-leishmanial, and cancer activities (Naik et al. 1988). It is also purified from *Fusarium proliferatum* (MTCC 9690), an endophyte associated with *Dysoxylum binectariferum* (Kumara et al. 2012). Rohitukine was also reported from species of *Fusarium* associated with *D. binectariferum* and *Amoora rohituka* (Kumara et al. (2014)

Rohitukine is reported to possess anticancer property. An extract of rohitukine producing fungus *Fusarium proliferatum* (MTCC 9690) has shown cytotoxicity against HCT-116 and MCF-7 human cancer cell lines at the IC_{50} of = 10 μg/ml (Kumara et al. 2012). It has been reported that rohitukine exhibits anticancer activity via modulating apoptosis pathways in A 549 cell line and yeast mitogen-activated protein kinase (MAPK) pathway (Safia et al. 2015). Flavopiridol, a semisynthetic derivative of rohitukine, is a broad-spectrum cyclin-dependent kinase (CDKs) inhibitor which entered in phase III clinical trials (Kelland 2000). Apart from anticancer property, rohitukine also possesses potent anti-hyperlipidemic activity (Chhonker et al. 2015).

19.1.8 Piperine

Piperine is an alkaloid of *Piper longum* and *Piper nigrum* plants of the Piperaceae. Piperine has been isolated from endophytic fungi *Periconia* sp. associated with *P. longum* (Verma et al. 2011), and *Mycosphaerella* sp. (Chithra et al. 2014a) and *Colletotrichum gloeosporioid* (Chithra et al. 2014b) from *P. nigrum*. Piperine possesses broad bioactive properties ranging from antimicrobial, antidepressant, anti-inflammatory, antioxidative, antimycobacterial, antihyperlipidemic, immunoregulatory, and antitumor properties (Sunila and Kuttan 2004; Maneesai et al. 2012; Huan et al. 2015). Interestingly, piperine also plays a vital role in increasing the bioavailability of many drugs.

Piperine possesses significant anticancer property. It inhibits the proliferation and survival of a wide range of cancer cells through its positive effect on apoptosis and inhibition of cell cycle progression. Piperine modulates the activity of many enzymes and transcription factors which led to the inhibition of invasion, metastasis, and angiogenesis. In addition, piperine is an inhibitor of para-glycoprotein (P-gp) and can reverse multidrug resistance (MDR) in cancer cells (Doucette et al. 2013; Rather and Bhagat 2018).

19.1.9 Hypericin

A naphthodianthrone derivative hypericin was purified from *Hypericum perforatum* (St. John's Wort), traditionally used to treat depression and other ailments (Brockmann et al. 1939; Nahrstedt and Butterweck 1997). Cytotoxic activity of hypericin is due to its photodynamic activity (Hadjur et al. 1996; Delaey et al. 2001; Kamuhabwa et al. 2001). Kusari et al. (2008) isolated hypericin along with emodin from an endophytic fungus *Thielavia subthermophila* associated with *Hypericum perforatum*, collected from India. The fungal extract containing both the compounds exhibited photodynamic cytotoxicity against the human acute monocytic leukemia cell line (THP-1) in two different assays. THP-1 cells were exposed to varying concentrations of the fungal extract in dark and after the extract had been irradiated with visible light for 20 min. In the resazurin-based assay, dark vs. light cell viability was 92.7% vs. 4.9%, and in the ATPlite assay, dark vs. light cell viability was 91.1% vs 1.0% (Kusari et al. 2009c).

19.1.10 Solamargine

Solamargine, a steroidal alkaloid in *Solanum nigrum*, exhibited potent anticancer activity against colon, prostate, breast, hepatic, and lung cancer cell lines (Hu et al. 1999; Liu et al. 2004; Mohy-Ud-Din et al. 2010). It was reported from an endophytic fungus *Aspergillus flavus* SNFSt associated with *Solanum nigrum* L. collected from Beni-Suef, Egypt. Solamargine production could be followed and quantified for a total of 11 generations of this fungus with a titer of ~250–300 μg l^{-1} (El-Hawary et al. 2016).

Solamargine has been found as the most active anticancer compound of *Solanaceae* plant family and showed broad-spectrum anticancer activity. It displayed cytotoxic activity against Hep3B, A549, HBL-100, MCF-7, K562, KB PC3, U2OS, B16F10, HT29, HeLa, MO59J, U343, U251, GM07492A, HL-7702, and RPE1 cell lines. Its mechanisms of action for anticancer activity include induction of apoptosis, cell cycle arrest, inhibition of cancer cell migration, and invasion (Kalalinia and Karimi-Sani 2017; Xie et al. 2017). Apart from anticancer activity, solamargine also displayed leishmanicidal activity against promastigote forms of *L. amazonensis* (Abreu Miranda et al. 2013).

19.1.11 Berberine

Berberine is an isoquinoline quaternary alkaloid (or a 5,6-dihydrodibenzo[a,g] quinolizinium derivative) isolated from medicinal plants such as *Hydrastis canadensis*, *Berberis aristata*, *Coptis chinensis*, *Coptis rhizome*, *Coptis japonica*, *Phellondendron amurense*, and *Phellondendron chinense* schneid (Imanshahidi and Hosseinzadeh 2008). Berberine displays various biological properties, viz., cardioprotective, antidiabetic, antibiotic, and antitumor (Sun et al. 2009). Berberine was isolated from *Alternaria* sp. BBH6, an endophytic fungus associated with *Phellodendron amurense* (Li et al. 2009). Later on, it was obtained from *Fusarium solani* isolated from roots of the *Coscinium fenestratum.* The yield of berberine was 196 µg/L (Vinodhini and Agastian 2013). It was also isolated from *Phomopsis*HL-BC, an endophyte associated with *Coptis chinensis* with the yield of 0.664 mg/L (Li et al. 2013). *Alternaria* sp. HL-Y-3, isolated from the leaves of *Coptis chinensis*, was also the source of berberine. The yield of berberine was recorded as 9.313 µg/L (Zhang et al. 2016). Hong and Yang (2010) optimize the culture condition of the strain S-NU-3-2 for berberine production which includes the optimal basic medium, carbon source, nitrogen source, illuminating condition, and culture temperature; there was an increase in the berberine yield by 47.2% compared with those under original culture conditions.

Berberine showed diverse biological activities such as antimicrobial, anti-inflammatory, inhibition of smooth muscle contraction, inhibition of ventricular tachyarrhythmias, elevation of platelet count in patients with primary and secondary thrombocytopenia, and stimulation of bile secretion and bilirubin discharge (Birdsall and Kelly 1997). There has been a growing interest for the use of berberine in the treatment of diabetes and obesity. Berberine activates the AMP-activated protein kinase (AMPK) which regulates an array of biological activities that normalize lipid, glucose, and energy imbalances (Lee et al. 2006). It also possesses cholesterol and triglycerides lowering properties (Kong et al. 2004).

19.1.12 Swainsonine

Swainsonine is a trihydroxy inolidizine alkaloid, a glycosidase inhibitor reported from a number of plant species, such as *Swainsona canescens*, *Ipomoea* spp.,

Turbina cordata, *Sidacarpinifolia*, and locoweeds (*Astragalus* and *Oxytropis* spp.) (Cook et al. 2014). It was also extracted from an endophytic fungus *Fusarium tricinctum* associated with *Oxytropis kansuensis* and *O. deflexa* in China (Lu et al. 2012). Recently, it has been reported from endophytic fungus *Undifilum oxytropis* isolated from locoweeds (Ren et al. 2017).

Swainsonine possesses significant anticancer activity against a broad range of cancers including gastrointestinal and pancreatic cancers. A study on the effect of swainsonine on human oesophageal squamous cell carcinoma cells showed that swainsonine promotes apoptosis in carcinoma cells through activation of mitochondrial pathway (Li et al. 2012b). Swainsonine inhibits the viability of human hepatoma cells (MHCC97-H) in a dose- and time-dependent manner. It significantly suppressed MHCC97-H cell growth by triggering cell cycle arrest at the G0/G1 phase and the induction of apoptosis (You et al. 2012).

19.1.13 Withanolide

Withanolide, the secondary metabolite from *Withania* species, has potential use in treating cardiovascular, Alzheimer's disease, etc. For improved industrial commercialization, there is a need to increase its production. Withanolides were purified from the roots and leaves of *Withania somnifera* (Sabir et al. 2013; Sangwan et al. 2014). Recently, it is identified from an endophytic fungus *Taleromyces pinophilus* isolated from leaves of *Withania somnifera*, which produces withanolides in the medium. The fungus produces a high amount of withanolide when compared to leaf and root of *W. somnifera* (Sathiyabama and Parthasarathy 2018).

Withanolides are a group of C28 steroids built on an ergostane scaffold functionalized at carbons 1, 22, and 26, commonly known as the withanolide skeleton (Chen et al. 2011). The extracts from *Withania somnifera* containing withanolide have been used for over 3000 years in traditional Ayurvedic and Unani Indian medical systems as adaptogenic, diuretic, anti-inflammatory, sedative, anxiolytic, cytotoxic, antitussive, and immunomodulatory. After the isolation and characterization of withanolides in chemically pure form, extensive research focusing on the pharmacological profiling of withanolides has been carried out and reported. Withanolides exhibit excellent anti-inflammatory property and have shown a beneficiary effect in inflammation-mediated chronic diseases such as arthritis, neurodegenerative and autoimmune disorders, neurobehavioral, and cancer. Excellent pharmacological activity with minimal side effects is the most beneficial feature of withanolide to be used in various neurodegenerative disorders (Chen et al. 2011; White et al. 2016).

19.1.14 Gastrodin

Gastrodin was isolated (57 μg/g) from mycelia of *Chaetomium cupreum* strain TMDF101, *Fusarium oxysporum f. sp. cyclaminis* strain TMBY302 at 89 μg/g, and *Armillaria cf.sinapina* strain TMSC108 at 184 μg/g associated with *Gastrodia elata*

(Su et al. 2014). Gastrodin, a phenolic glycoside, is the major constituent of the Chinese herb *Gastrodia elata* Blume. *Gastrodia elata* Blume has been used traditionally in the treatment of headache, dizziness, spasm, epilepsy, stroke, and amnesia and as an analgesic since ancient times. Gastrodin has been investigated for its use in wide range of central nervous system disorders such as epilepsy, Alzheimer's disease, Parkinson's disease, cerebral ischemia, and cognitive impairment (Liu et al. 2018). Xiao et al. (2016) reported that gastrodin protects against chronic inflammatory pain by depressing spinal synaptic potentiation via blockade of acid-sensing ion channels (Xiao et al. 2016). It also possesses antioxidant and anti-inflammatory activities.

19.1.15 Gymnemagenin

The triterpenoid gymnemagenin was reported from *Gymnema sylvestre* plant, which is used in the pharmaceutical industry as an antidiabetic, anti-obesity, and antiviral agent (Liu et al. 1992; Kanetkar et al. 2007; Rao and Sinsheimer 1971). It is also purified from an endophytic fungus *Penicillium oxalicum* isolated from the leaves of *Gymnema sylvestre* (Parthasarathy and Sathiyabama 2014). Studies on gymnemagenin suggest that it can be used in the management of diabetes and obesity (Pothuraju et al. 2014; Rathore et al. 2016).

19.1.16 Chrysin

Chrysin (5,7-dihydroxy flavone, ChR) is a C-glycosyl flavonoid of *Passiflora incarnata* (Blumenthal et al. 2000), possessing numerous biomedical properties, such as antibacterial (Wang et al. 2011c), anti-inflammatory (Gresa-Arribas et al. 2010), antidiabetic (Torres-Piedra et al. 2010), anxiolytic (Brown et al. 2007), hepatoprotective (Pushpavalli et al. 2010), anti-aging (Anand et al. 2012), anticonvulsant (Medina et al. 1990), and anticancer (Khoo et al. 2010) effects. Accordingly, it actively inhibits inflammatory enzymes such as iNOS and COX-2 by inducing the PPAR (Liang et al. 2001). Recently, chrysin was purified from *Alternaria alternata* KT380662, *Colletotrichum capsici* KT373967, and *Colletotrichum taiwanense* PI-3 KX580307, the endophytes associated with the leaves of *Passiflora incarnata*. The maximum production of ChR was observed in the *A. alternate* KT380662 with rates measuring approximately 846 mg/L (Seetharaman et al. 2017).

19.1.17 Sanguinarine

Sanguinarine, a benzophenanthridine, is reported from many plants, including *Argemone mexicana*, *Chelidonium majus*, *Macleaya cordata*, bloodroot *Sanguinaria canadensis*, *Bocconia frutescens* and *Bocconia frutescens* (Papaveraceae), and

Poppy fumaria (Fumariaceae) [Santos and Adkilen 1932; Mackraj et al. 2008]. *Chelidonium majus* is found in Egypt (Khayyal et al. 2001) and in South Africa (Panzer et al. 2000).

Sanguinarine possesses antibacterial, anthelmintic, and anti-inflammatory properties (Weerasinghe et al. 2013). Sanguinarine was also purified from fungus *Fusarium proliferatum* BLH51, an endophytic residing inside the *Macleaya cordata* plant that was the source of sanguinarine. The amount of sanguinarine produced by this endophytic fungus was quantified to be 178 μg/L, substantially lower than that produced by the host tissue (Wang et al. 2014c).

Sanguinarine has shown potent anticancer activity against a wide range of cancer cell lines such as lung, colon, breast, pancreatic, skin, cervical, prostate, and hematological malignancies. Its anticancer mechanisms include induction of apoptosis via extrinsic and intrinsic apoptotic pathways, induction of cell death via bimodal cell death (oncosis), inhibition of tumor cell invasion (antimetastasis), and inhibition of cancer cell angiogenesis. In addition, Sangguinarine also sensitizes the cancer cells to the standard chemotherapeutics which led to the enhanced cytotoxic effects (Achkar et al. 2017; Fu et al. 2018).

19.1.18 Cajanol

Cajanol (5-hydroxy-3-(4-hydroxy-2-methoxyphenyl)-7-methoxychroman-4-one) is an isoflavanone from *Cajanus cajan* (Pigeonpea) roots and a cytotoxic compound (Luo et al. 2010). Cajanol was also purified from *Hypocrea lixii* and endophyte associated with *C. cajan.* R-18 produced the highest levels of cajanol (322·4 μg/l or 102·8 μg/g dry wt. of mycelium) after incubation for 7 days. Fungal cajanol possessed strong cytotoxicity activity toward A549 cells with IC_{50} value of 20.5 μg/ml after 72 h treatment. The cajanol exhibited toxicity toward normal cells MC3T3-E1 cells and RAW264.7 with IC_{50} value of 48.7 and 40.2 μg/ml, respectively, after 72 h of treatment (Zhao et al. 2013b).

19.1.19 Azadirachtins

Azadirachtins, belonging to the limonoids, are tetranortriterpenes reported from members of Rutaceae and Meliaceae group (Isman 2006). Azadirachtins A and B were purified from *Penicillium* (*Eupenicillium*) *parvum*, an endophytic fungus associated with *Azadirachta indica* (Kusari et al. 2012). Three unidentified strains endophytic in *M. azedarach* have been found to produce this compound (Wang et al. 2007). Its antitumor property is also envisaged (Man et al. 2012).

Azadirachtin exhibits deleterious effects on phytophagous insects. It disrupts insect molting by antagonizing the effects of ecdysteroids. An antifeedant (feeding deterrent) is a chemical which inhibits feeding by insect and is used in crop

protection. Azadirachtin possesses strong antifeedant property with a wide range of insects and desert locust *Schistocerca gregaria*. Azadirachtin is deterrent to *S. gregaria* at 0.04 ppm. It also possesses excellent insecticidal activity. Azadirachtin has been tested on more than 600 insect species and showed insecticidal activity at 1–10 ppm (Morgan 2009).

19.2 Approaches for Overcoming Challenges Associated with Endophyte Natural Products

Despite valuable pharmaceutical, nutraceutical, and industrial potential, endophyte based molecular pharming are not commercially successful so far. Although numerous molecules form endophytes possessing valuable pharmaceutical, nutraceutical, and industrially important properties have been commercialized, there are new hope and potential for the synthesis of novel and high-value bioactive compounds from endophytic fungi. As discussed above, several independent research groups have successfully shown in vitro production of natural products. Limited success has been obtained for the industrial production of such compounds. Several practical bottlenecks such as low yield, lack of data on the exact nature of fungi-plant biochemical and molecular interaction that leads to natural product synthesis, scale-up problems, and culture in the axenic state limit its success. Further, there is a lack of understanding of how the interaction between endophytic fungal organism and other microbes interplays. Various ideas have been tested to overcome such problems. For instance, culturing the host tissues in vitro has not achieved much success due to genetic instability of tissue culture propagated hosts, slow fungal growth, formation of cell clumps, and prone toward shearing. Further, tissue culture maintenance is more laborious and is not economical compared to the fomentation-based processes. On the other hand, fermentation-based optimization of plant natural product synthesis failed to reach an industrially sustainable level of production as obtained in vivo inside the host tissues. The main implications of endophytic fungus-based product development using these two approaches remain to be seen in the future.

Further, endophytic fungi do not continue to produce target metabolite due to continuous subculturing and axenic culture maintenance. Therefore, it is important to restore the active metabolite production state of endophytes for pharmaceutical usage. This can be accomplished by a multipronged approach encompassing comprehensive understanding of the dynamic endophyte(s)-host communications affecting their biological, biochemical, and genetic characteristics. Further, as plant acts as a host to the endophytes, it substantially influence the *in planta* biosynthetic process of the fungus. Imperical evidence from the whole genome sequencing information has demonstrated that a number of known bioactive molecule in fungus and bacteria are significantly higher compared to their number of genes contributing toward the biosynthetic enzymes of these microbes (Winter et al. 2011). Therefore, any small variation in culture condition or host metabolism has a huge impact on endophyte secondary metabolism (Scherlach and Hertweck 2009). A systematic

understanding of the endophyte-endophyte and endophyte-host interspecies interactions is essential for the sustainable production of target metabolite using endophyte-based production systems (Kusari and Spiteller 2011).

Media optimization and supplementation and use of appropriate elicitors could address the limitation associated with a low yield of endophyte natural products. An attractive approach is to trigger specific genes for augmenting target natural product. To achieve this, combinatorial chemical synthesis approaches may not be fruitful due to the complex structural features of the metabolites. However, inducer/elicitor of the specific biochemical pathway has been tested with moderate success. Alternatively, use of signaling molecules and economical precursor feeding for culturing endophytes has been tried. These precursors may act as effective triggers for biochemical pathways to achieve higher metabolite yield. There are concerns regarding the accumulation of several unwanted metabolites that can potentially hinder enzyme action in the culture medium. However, this needs a critical investigation to tackle negative feedback mechanism associated with the fungal fermentation process.

Several newly developed approaches could help in enhancing the desired natural product from endophytes. These include genetic engineering, RNAi, genome editing, genetic transformation, mutagenesis, and gene cluster identification. The most common strategy of metabolic engineering includes the inactivation of the competing pathways through gene deletion and/or augmentation of cooperative pathways of the target natural product amplification for target natural product overproduction. The potential metabolic engineering intervention for biosynthesizing plant metabolites in endophytic fungi has undoubtedly been recognized. However, there is still no remarkable development in the biotechnological synthesis of these bioactive secondary metabolites using endophytes. It is therefore key to understand the metabolome in endophytes correlating to their host plants on a case-by-case basis to comprehend how the biosynthetic gene clusters are controlled and their expression is regulated *in planta* and ex planta (by environmental factors and axenic cultures). A clear mechanistic elucidation of the host-endophyte molecular and genetic interaction will help to fine-tune and enhance specialized metabolite production under commercial production systems. Genetic engineering could potentially manipulate the plant metabolite biosynthesis within endophytes to design novel lead structures that could act as pro-drugs. Further, genome mining of diverse endophyte strains could significantly impact the manipulation of novel specialized metabolites. The endophytes that are unculturable under axenic conditions could be recovered by environmental PCR combined with culture-independent metagenomic strategies. Structure-guided gene targeting can be used for biosynthetic gene clusters (Kusari and Spiteller 2011). Comprehensive understanding of unculturable endophytes and their biosynthetic process will allow heterologous expression of the target product in model microbes like *Saccharomyces cerevisiae* or *Escherichia coli*.

An additional benefit of endophytic fungi over hosts is that the biosynthetic gene modules of a metabolite might be synthesized by multiple interdependent biosynthetic steps organized as an operon in adjacent clusters in the compact fungal genome. This could permit a quick in silico prediction of signature genes or

characterization of protein domains that are specific to target metabolic pathway. This characteristic of endophytic genome architecture enhances the possibility of expressing gene cluster-based complete metabolic pathways in heterologous organisms. We are facing a unique acute challenge where global biodiversity is diminishing at a frightening rate. Many endemic medicinally important host plants are endangered and are on the verge of extinction, so the endophytes lived inside them are also disappearing. Therefore, a completely new approach must be implemented to understand the fundamental molecular basis of metabolite biosynthesis to safeguard a constant and sustainable supply of bioactive pro-compounds for the existing and emerging diseases. Some of the new technologies can be implemented in this direction as described below.

Endophyte-mediated production of complex bioactive compounds is the outcome of the direct or indirect interaction of host and environmental factors with the fungus. Recent advances in high-throughput omics technologies have begun to give a comprehensive understanding of plant-endophyte interactions. Next-generation sequencing technologies can be used for transcriptomics studies such as gene discovery, transcript quantification, pathway representation, comparative analysis, and micro-RNAs and marker discovery for non-model plant and its endophyte species without a fully annotated genome. Predominant classical biotechnological approaches, however, were unsuccessful in unraveling complete monitoring of reciprocal molecular network regulations for high-value natural products. The next-generation metabolic engineering approaches are providing tangible evidence on the molecular-genetic and biochemical components of the host-endophyte interactions. Metabolic modeling in combination with systems biology has started to give high-resolution data on host-endophyte mutualist interactions. However, translating these fundamental biological findings into industrial production of bioactive from endophytes still remains a difficult task. Genetic engineering and heterologous production of endophyte natural products are also very challenging with our current understanding. It is practically tough to engineer orthogonal metabolic pathways which are unlinked to the target endophyte metabolic network and bypass unwanted molecular interactions which have undesirable outcomes. This task is further complicated if the target biomolecule is produced through a multistep, cascade pathway. As of now, we have very limited knowledge on sophisticated aspects of molecular pathway induction and modulation, flux distribution, subcellular level regulations in biosynthesis, and storage and discharge of target metabolite. Complete understanding of these aspects is very crucial for heterologous genetic engineering to be successful. Interdisciplinary efforts are essential for the sustainable industrial production of these target metabolites.

Future endophyte research also should emphasize to have a clear regulatory guideline on bioactive natural products that are safe for human consumption along with plant and environment. Therefore, the major focus should be on finding a safe bioactive natural product with therapeutic properties. Further, to enhance the specificity and efficacy of drug molecule, emphasis should be given on modifying the structure of biomolecules. The fermentation condition should be optimized such a way that the target molecule should show maximum bioactivity and yield. The

focused gene discovery programme should be taken in mission mode in order to identify and characterize the target endophyte gene cluster. This will not only identify the rate-limiting and regulatory steps in a metabolic pathway; it can also lead to the heterologous engineering of bioactive molecule in fomentation-compatible model microbial systems.

19.3 Conclusions

Present data reveal that there is increasing number of bioactive compounds produced by endophytic fungi which were primarily produced by plants. This clearly indicates that there is a potential in these fungi for further applications. For instance, in the case of swainsonine biosynthesis, direct evidence suggests that some of the plant metabolites can be actually synthesized by their associated endophytic fungi due to mutual genetic exchange between the host plant to endophytic fungi. In some cases, endophyte-mediated metabolites are produced in very small quantity. In many instances, after a product has been accumulated, the fungus may inhibit the synthesis of the desired compound(s).To overcome these challenges, various techniques are used, which include optimization of media using OSMAC, RSM, etc. In some cases, epigenetic modifiers like HDAC and DNMT inhibitors are used to overcome the problem of attenuation. Recently, the high-throughput omics technologies are being implemented to further understand the host-endophyte relationship for producing target compound(s). The detailed understanding of the molecular-genetic regulation of endophyte gene clusters for specialized metabolism will accelerate the heterologous metabolic engineering of bioactive target molecules in a sustainable manner for the industry.

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20 Wild Edible Mushrooms of North West Himalaya: Their Nutritional, Nutraceutical, and Sociobiological Aspects

N. S. Atri, Y. P. Sharma, Sanjeev Kumar, and Mridu

Abstract

North West Himalayan region is quite rich in mushroom microbiota. Many of their species are edible, few are hallucinogenic, and some are poisonous. The edible ones are being regularly collected during monsoon season year after year from the nearby forested areas by the local inhabitants mostly for their personal consumption as well as for trading so as to earn some additional money for meeting their day-to-day expenditure. Several such species either belong to ascomycetous fungi (e.g., *Tuber* P. Micheli ex F.H. Wigg., *Morchella* Dill. ex Pers., *Geopora* Harkn., etc.) or agaricomycetous fungi (e.g., *Clavaria* L., *Ramaria* Fr. ex Bonord., *Hericium* Pers., *Sparassis* Fr., *Cantharellus* Juss., *Russula* Pers., *Lactarius* Pers., *Volvariella* Speg., *Pleurotus* (Fr.) P. Kumm., *Lentinus* Fr., *Amanita* Pers., *Termitomyces* R. Heim, *Macrolepiota* Singer, *Lycoperdon* Pers., *Boletus* L., etc.). For some of them, especially morels, there is an organized trade syndicate which works right from Jammu and Kashmir to Uttarakhand in the North West Himalaya. Many of these mushrooms are being evaluated for their nutritional and nutraceutical constituents. The results of the analysis have revealed these mushrooms to be quite rich in proteins, carbohydrate, and important minerals, while their fat content is substantially low making them a low-energy or low-calorie functional food. From nutraceutical point of view, it has been found that these are quite rich in vitamins, phenols, flavonoids, steroids, carotenes, lycopenes, alkaloids, and a number of specific bioactive metabolites with extreme therapeutic relevance. Because of their significance in human welfare and ecosystem maintenance and replenishment, mushrooms are rightly treated as special creation of God in the nature's treasure.

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20.1 Introduction

Nature has blessed the humankind with a number of endowments, and mushrooms are one of them. In view of the associated benefits, mushrooms are recognized as incredible creation of God. In the scientific terms, mushrooms refer to a large group of macro fungi growing above the ground that emerges out from the belowground mycelium through a fruiting process (Chang and Miles 2004; Kalac 2009). In general, mushrooms represent ascomycetous and agaricomycetous fungi mostly forming aboveground sporophores except truffles which are underground. It is estimated that around 1.5 million species of fungi exist in nature and only about 10% of them are known at present (Chang and Miles 2004; Sullivan et al. 2006; Wasser 2011). Large majority of the undiscovered species are assumed to be of possible benefit to mankind in one way or the other. Mushrooms are known to inhabit a variety of ecological niches such as ground or soil (terrestrial), living plants or logs (lignicolous), decaying leaf litter (humicolous), animal dung (coprophilous), symbiotic association with plant roots (mycorrhizal) and insects (entomogenous), or even underground (Singer 1986). Collection of wild mushrooms is a common practice around the globe since ancient times (Wani et al. 2010a, b). They possess a variation of reputations in the society beginning from edible, inedible, curative, poisonous, decorative, and even to the mythological terms as magical and mysterious (Pegler 1977; Purkayastha and Chandra 1985; Singer 1986; Rai and Arumuganathan 2005). Mushrooms have been a choice source of food as well as medicine for Greeks, Egyptians, Chinese, Mexicans, Indians, and Romans from their early civilizations. Besides, mushrooms have got an important place in relation to the sacred beliefs as well. The Romans treasured mushrooms as “food of gods,” the Chinese considered them as the “elixir of life” due to their benefits for health, the Pharaohs consumed them for delicacy, and the Greeks believed that they provided strength to the soldiers during wars (Chang and Miles 2004; Zhang et al. 2014).

An assortment can be found in the morphology of the mushrooms, be it their color, shape, presence or absence of veil, spore color, etc. Anatomically also they differ in the shape and size of spores, basidia, cystidia (if present), hyphal system, etc. On the basis of all these morpho-anatomical characters as well as reactions of the basidiospores/basidiocarp parts with various reagents, mushrooms are being characterized since the times of Fries (1821), Kühner (1980), and Singer (1986). In the modern times, systematic studies have attained a higher level with synthetic approach including application of molecular tools (Atri et al. 2017). Now molecular techniques have become an important tool for studying taxonomic and phylogenetic relationships among various fungi including mushrooms (Zambino and Szabo 1993). Molecular markers of rDNA sequencing, RFLP (Restriction Fragment Length Polymorphism), RAPD (Random Amplified Polymorphic DNA), microsatellite, and mitochondrial genotypes are being used in taxonomic studies of various

mushroom species including *Agaricus* L. (Castle et al. 1987; Sonnenberg et al. 1991; Khush et al. 1992; Barroso et al. 2000; Moore and Chiu 2001; Ramirez et al. 2001) and *Volvariella* Speg. (Chiu et al. 1995).

Besides their vivid morphological range, some of the delicious species of ascomycetous and agaricomycetous fungi including *Morchella* Dill. ex Pers., *Tuber* P. Micheli ex F.H. Wigg., *Ramaria* Fr. ex Bonord., *Sparassis* Fr., *Cantharellus* Juss., *Agaricus* L., *Flammulina* P. Karst., *Pleurotus* (Fr.) P. Kumm., *Laetiporus* Murrill, *Lentinus* Fr., *Lentinula* Earle, *Volvariella* Speg., *Macrolepiota* Singer, *Termitomyces* R. Heim, *Calocybe* Kühner ex Donk, *Amanita* Pers., *Russula* Pers., *Lactarius* Pers., *Boletus* L., and *Lycoperdon* are preferred for culinary purposes. Their culinary and commercial value is mainly due to their organoleptic properties such as their aroma, good taste, acceptable flavor and other such features including texture and digestibility (Valverde et al. 2015; Atri et al. 2016; Kalac 2016). Their nutritional status is also supported by the scientific data which have been given by a number of workers over a past few decades. Proximate composition, amino acid profile, vitamins, minerals and others have been analyzed for many wild and cultivated species. They are reported to contain a rich amount of high-quality digestible proteins and carbohydrates, low fat content, and a moderate level of ash and crude fibers (Aletor and Aladetimi, 1995; Alofe et al. 1996; Atri et al. 2013, 2016; Lalotra et al. 2016; Mridu and Atri 2017). They are also a good source of minerals, vitamins, and essential amino acids (Crisan and Sands, 1978; Breene 1990; Bobek et al. 1995; Bobek and Galbavy 1999; Manzi et al. 1999, Atri et al. 2010, 2016; Mukhopadhyay and Guha 2015). Like higher plants, macroelements, viz., potassium, phosphorus, sodium, calcium, and magnesium, as well as microelements, including copper, zinc, iron, molybdenum, selenium, and cadmium, are also present in sufficient quantities in edible mushrooms (Bano et al. 1981; Bano and Rajarathanam 1982; Li and Chang 1982; Atri et al. 2016). Their nutritional value is comparable to many other vegetables, fruits and dairy products, etc. where mushrooms are reported to excel in comparison (Crisan and Sands 1978; Chang and Miles 2004). Because of the presence of important bioactive substances, mushrooms nowadays are counted in the category of "nutraceuticals," which means a food containing concentrated forms of bioactive agents in higher quantities as compared to that of a normal food. In view of these qualities, even Food and Agricultural Organization (FAO) has suggested their usage as food supplement for protein-deficient and undernourished population of developing and underdeveloped countries.

Wild edible mushrooms also offer plethora of therapeutic and health benefits in addition to essential nutrients (Brower 1988; Zeisel 1999; Atri et al. 2012a,b;Kumari et al. 2013; Kumari and Atri 2014; Sheikh et al. 2015). Many of the mushroom species contain biologically active polysaccharides which provide immunostimulatory and antioxidative properties (Chang and Buswell 1996; Ikekawa 2001). There are many substances known to be present in them having specific therapeutic properties. Lectins, reported in *Agaricus campestris* L., *Volvariella volvacea* (Bull.) Singer (Volvatoxin), *Flammulina velutipes* (Curtis) Singer, and *Pleurotus ostreatus* (Jacq.) P. Kumm. (Pleurotolysin), have shown antitumor, immunomodulatory, and hemolytic activities (Sage and Vazquez 1967; Lin et al. 1973; Bernheimer and Avigad 1979; Ganguly and Das 1994; Wang et al. 1995, 1996, 1997).

Wild edible mushrooms are also a rich repository of secondary bioactive metabolites such as phenolic compounds, flavonoids, terpenes, steroids, etc. which have gained importance due to their large array of biological actions. Among these, in particular, phenols account for free radical scavenging, metal chelation, and enzyme modulation, thereby conferring antimicrobial, antioxidant, anticancerous, anti-inflammatory, and antiviral properties (utility in effective treatment of HIV infections) (Nanba 1993; King 1993; Wasser and Weis 1999; Hirano et al. 2001; Cheung and Cheung 2005; Puttaraju et al. 2006; Sarikurkcu et al. 2008; Ferreira et al. 2009; Alves et al. 2012; Kumari and Atri 2012; Reis et al. 2012; Finimundy et al. 2013; Yildiz et al. 2015). These are also reported to be quite effective against stress, insomnia, asthma, allergies, and diabetes (Bahl 1983). In recent years, side effects and toxicity of synthetic products and medicines are bringing back the interest of people and scientists toward natural health foods (Fukushima and Tsuda 1985; Stone et al. 2003). Therefore, mushrooms are appreciated as one of those foods which can help in preventing many deadly diseases if added in one's routine diet.

In addition to the health benefits provided by the mushrooms, they constitute the most relished food commodities among the number of nonconventional foodstuffs primarily because of their unique flavor and texture. The edible wild mushrooms are most important in food security of ethnic groups and tribals and also a good source of income to the native people throughout the world (Sysouphanthong et al. 2010). Though many species of mushrooms are being cultivated and sold in markets, still the traditional practice of collecting wild mushrooms for nutritional and medicinal purposes continues in India and many other countries. This indigenous knowledge of wild edible fungi and their utilization by local population is an important component of ethnomycology. Documentation of the indigenous knowledge of wild edible mushrooms has been done by different researchers from the NW Himalaya, and these are popular by various folk names in different areas, and their consumption patterns also vary (Atri et al. 2005, 2012a; Wani et al. 2010a; Kumar and Sharma 2011; Yangdol et al. 2014; Dorjey et al. 2017). However, their proper identification and toxicity studies are important to avoid accidents and to assure safe use as some species are poisonous too.

20.2 Physiognomy, Climate, and Mushroom Diversity

North West Himalayan region represents the youngest mountain systems of the world comprising many highest peaks. Geographically this area lies between 29° 03′–34° 03′ N latitude and 74° 02′–79° 05′E longitude. The vast range of topographical features and climate witness a great diversity of flora and fauna in this region (Ray et al. 2011).These mountain ranges are spread over the three States in India, viz., Jammu and Kashmir, Himachal Pradesh, and Uttarakhand (Champion and Seth 1968).There is a drastic altitudinal variation in Himalayas beginning from 300 m and rising till 8400 m and above (Wanchoo 2000). The soil of this region is slightly acidic to neutral and contains medium to high level of organic matter. Annual rainfall ranges from 600 mm to 3200 mm, and most of the rains occur in the

months of July to September. Temperature also varies greatly with the altitude; it decreases with an increase in the altitude (Puri 1960). The climate and physiognomic features along with vegetation composition of the area have direct bearing on the mushroom diversity. Many of the mushrooms which form symbiotic association with the nearby trees form interconnecting wood wide web which plays a major role in the flow of nutrients including minerals thereby helping in the improvement of plant health. The vegetation of North West Himalayan region is divided into various zones on the basis of climate with respect to altitudinal variations, viz., tropical, subtropical, temperate, subalpine, and alpine zone (Wanchoo 2000). The tropical zone constitutes the outer ranges of Shivaliks, where altitude ranges up to 1000m from the mean sea level. Tropical deciduous forests occurring in this zone consist of *Shorea robusta* C.F. Gaertn., *Dalbergia sissoo* Roxb., *Acacia catechu* (L.f.) Willd., *Bombax ceiba* L., *Cassia fistula* L., *Mangifera indica* L., *Ziziphus jujuba* Lam., etc. in addition to many shrubs and ferns. Many wild edible mushrooms including species of *Agaricus*, *Auricularia*, *Pleurotus*, *Volvariella*, *Lentinus*, *Macrolepiota*, *Termitomyces*, and species of *Tuber*, *Russula*, *Lactarius*, and *Amanita* are quite prominent in this region. Subtropical zone is an intermediate zone between tropical and temperate zones, extending from 1000 m to 1500m above mean sea level. Montane subtropical forests lie in this zone where hill tops and slopes are covered with pure belt of *Pinus roxburghii*. Other than this, *Quercus leucotrichophora* A. Camus ex Bahadur, *Lyonia ovalifolia* Hort., *Rhododendron arboreum* Sm., *Ficus palmata* Roxb., *Pyrus pashia* Buch.-Ham. ex D.Don, and others are also found there. Commonly occurring ferns of this area are *Dryopteris marginata* Christ, *Adiantum venustum* D.Don, *Pteris vittata* L., etc. Here also the edible species of mushrooms which are common in the tropical ranges of outer Himalayas can be prominently encountered. Besides puffballs, various species of *Morchella*, *Geopora*, *Rhizopogon*, and *Tuber* also appear prominently in this region. Above this occurs the temperate zone which extends from 1500 m to 3300 m above mean sea level. This region has been divided into lower oak zone (1500–2000 m), middle oak zone (2000–2500 m), and upper oak zone (2500–3300 m) [Champion and Seth 1968]. *Quercus leucotrichophora* is the prominent woody element present in the lower oak zone along with other tropical and temperate tree species, *Quercus dilatata* A. Kern. is a major species of middle oak zone, while *Quercus semecarpifolia* Sm. is dominant in upper oak zone. In this region various edible species of *Cantharellus*, *Russula*, *Lactarius*, *Lactifluus* (Pers.) Roussel, *Boletus*, *Amanita*, *Ramaria*, *Sparassis*, *Hericium* Pers., *Gyromitra* Fr., *Auricularia* Bull. ex Juss., etc. can be commonly encountered during the monsoon months. The subalpine zone falls between an altitudinal range of 3200–3500 m consisting of trees like *Abies pindrow* Royle, *A. spectabilis* (D.Don) Spach, *Quercus semecarpifolia*, *Betula utilis* D.Don, *Juniperus recurva* Buch.-Ham. ex D.Don, etc. The alpine zone extends from 3600 to 4500 m and above with bushy and short vegetation and trees like *Betula utilis*. Russulaceous mushrooms and Boletes are quite common in these areas.

20.3 Diversity of Common Wild Edible Mushrooms

Currently, the number of mushrooms estimated is 1,50,000 to 1,60,000 species, out of which approximately 16,000 have been described so far (Hawksworth 2001, 2012; Kirk et al. 2008; Zied and Pardo-Gimenez 2017). According to Chang and Miles (2004) around the world over 7000 mushroom species possess varying degrees of edibility and more than 3000 species spread over in 31 genera are regarded as prime edible. From India, about 300 edible species belonging to 70 genera are documented. In all, about 80 mushrooms have been grown experimentally, 20 cultivated commercially, and 4–5 species are being produced on a large scale (Chadha and Sharma 1995; Atri et al. 2009). Cultivated edible, medicinal, and wild edible mushrooms are the three major components of the global mushroom industry valued together at $63 billion in 2013. The cultivated edible mushrooms contribute the majority share of 54%, while wild mushrooms contribute only 8% to the total exchequer. Global production of mushrooms has also increased manifold from about 1 billion kg in 1978 to 34 billion kg in 2013 with China as the leading country with over 30 billion kg of mushroom production followed by European Union, America, and other countries with 3.1 billion kg production, while Asia produced 1.3 billion kg of mushrooms only (Royse et al. 2017). There are a large number of these mushrooms which are yet to be domesticated but are being collected by the local inhabitants in different parts of North West Himalaya during monsoon season (Fig. 20.7a–d). They fall in the categories of non-lamellate, agaricoid, and gasteromycetoid mushrooms. The non-lamellate mushrooms constitute a number of wild edible mushrooms including chanterelle, hedgehog mushrooms, coral fungi, morels, false morels, cauliflower fungi, truffles, false truffles, jelly fungi, polypores, boletes, etc. The lamellate mushrooms are categorized as agaricoid, lentinoid, pleurotoid, termitophilous, amanitoid, macrolepiotoid, russuloid, and volvarioid, while gastromycetoid mushrooms constitute *Podaxis* Desv. and puffballs. Some of these commonly gathered edible mushrooms are discussed with respect to their edibility, nutraceutical utility, and sociobiological aspects in the ongoing account.

20.4 Non-lamellate Edible Mushrooms

Chanterelle *Cantharellus cibarius* Fr. and *C. infundibuliformis* (Scop.) Fr. are edible fleshy species reported from the forests of Indian Himalayas (Sohi et al. 1964; Abraham et al. 1980; Beig et al. 2011 Kumar and Sharma, 2011). They are characterized by funnel-shaped carpophores which vary greatly in color and size. They commonly grow on exposed surface or under the surface of fronds of *Onychium* Kaulfuss fern (Lakhanpal 1994). *Albatrellus cristatus* (Schaeff.) Kotl.& Pouzar is yet another species growing on humicolous soil. It is eaten by the local population of Sangla Valley in Kinnaur District of Himachal Pradesh and is locally known as "Muh" (Sagar et al. 2017). It is characterized by yellowish- to orange-colored sporocarps with waxy margins. *C. cibarius* and *C. infundibuliformis* are known to be nutritionally and medicinally important mushrooms that are reported to contain a

favorable amount of secondary metabolites, amino acids, proteins, and abundant essential and nonessential minerals (Danell and Eaker 1992; Barros et al. 2008; Wani et al. 2010b; Drewnowska and Falandysz 2015).

Toothed Mushrooms Another group of non-lamellate mushrooms are toothed fungi also known as hydnums or Hedgehog mushrooms. *Hericium coralloides* (Scop.) Pers. or "Bear's head tooth" belongs to this category of macrofungi. It occurs on the branches of conifers in the form of a fascinating large whitish mass with small tufts of teeth. Its fresh fruit bodies are collected and splitted into small segments for consumption. They are also preserved by drying for use in off seasons (Lakhanpal, 1994). People of Garhwal Himalayas consider it as the best wild edible mushroom (Singh et al. 2017). Another wild edible species, *H. erinaceus* (Bull.) (Fig. 20.1a) Pers. is also consumed in this region (Singh et al. 2017). *Hydnum repandum* L. is still another excellent edible species which is commonly eaten by the people of Sungra region of Kinnaur District in Himachal Pradesh (Sagar et al. 2017).

Coral Fungi Coral fungi or Clavarias are another group of widely consumed edible mushrooms in Himalayas as well as other parts of India (Giri et al. 2012; Chauhan et al. 2014; Semwal et al. 2014). They are locally called as "Chinchhuru" which means "coral-like branched structures" (Fig. 20.1b). People of Himachal Pradesh call them "Siun" in some areas and "Chinmuh" in Kinnaur district (Sagar et al. 2017), and those of Uttarakhand refer them as "Ungli-Cheon" (Semwal et al. 2014). Commonly called as "Shairee" in Bhadarwahi and Gaddaishi languages in Jammu and Kashmir, *Ramaria apiculata* (Fr.) Donk, *R. aurea* (Schaeff.) Quél., *R. flavobrunnescens* var. *aurea* (Coker) Corner, *R. flavobrunnescens* var. *Longisperma* Kumar and Sharma, *R. formosa* (Pers.) Quél., and *R. stricta* (Pers.) Quél. are consumed in fresh form by the rural populace (Kumar and Sharma 2011). *Clavaria vermicularis* Sw. and *Clavulina alpine* S. Kumar & Y.P Sharma are also a part of their diet (Kumar and Sharma 2009). Most preferred among these mushrooms is *Ramaria botrytis* (Pers.) Ricken. It has cauliflower-like branched carpophores which are pinkish in color. It is also known for its antimicrobial activities (Giri et al. 2012). *Ramaria botrytoides* (Perk) Corner is a commonly consumed mushroom in the Sangla and Rakcham area of Kinnaur District of Himachal Pradesh. It is locally referred as "Kyalmangmuh" and is cooked with rice as Rice Pulao (Sagar et al. 2017).

False Morels Among the false morels, species of *Helvella* L. and *Gyromitra* Fr. (Fig. 20.1c, d) are the edible mushrooms of Himalayas (Sohi et al. 1965; Lakhanpal 1994; Chauhan et al. 2014). Among these, *Helvella crispa* (Scop.) Fr. (white form) is a widely consumed species. It has a white, saddle-shaped cap and fruited stalk. It occurs in both deciduous and coniferous forests, regenerating forested, woody, or

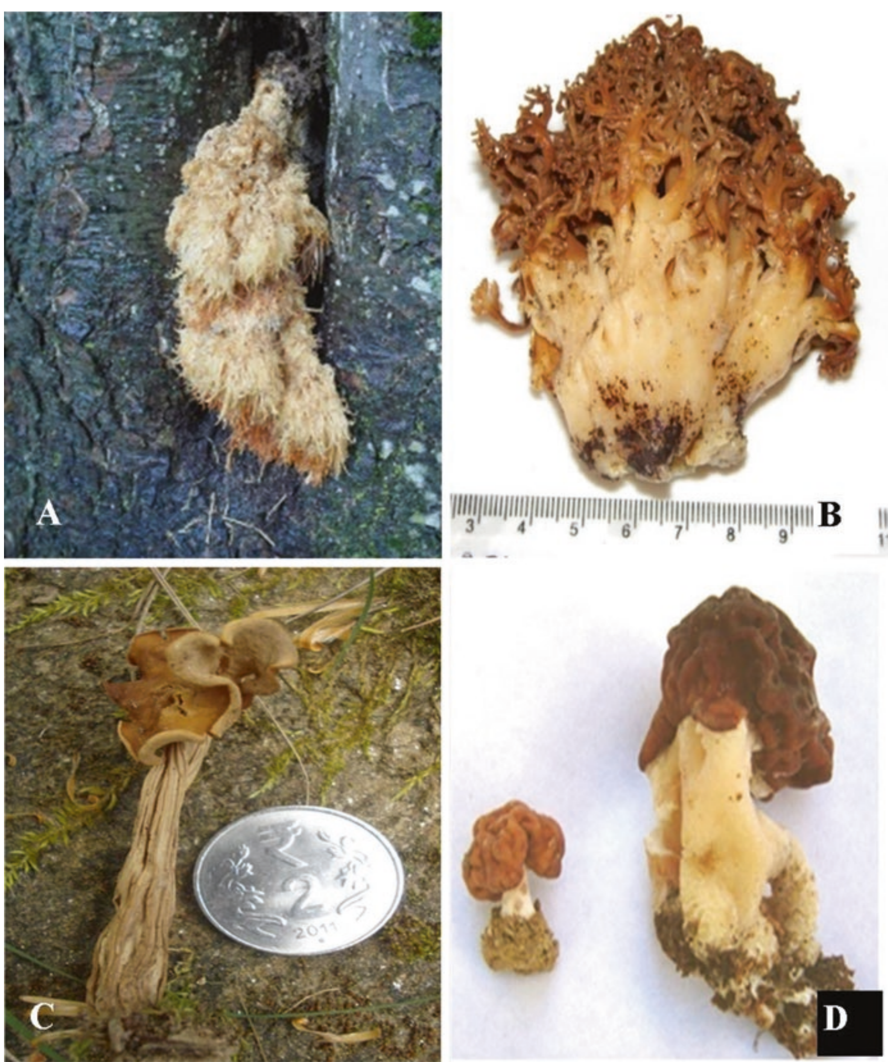

Fig. 20.1 Non-lamellate edible wild mushrooms of NW Himalayas. (**a**) *Hericium erinaceus*. (**b**) *Ramaria flavobrunescens*. (**c**) *Helvella elastica*. (**d**) *Gyromitra esculenta*

open grassy areas. In Kinnaur District of Himachal Pradesh, it is locally named as "Muh." Sporophores of this mushroom are reported to be edible when young. It is consumed as dry vegetable only after cooking (Sagar et al. 2017). In addition, *H. atra* Oeder, *H. elastica* Bull., *H. lacunose* Afzel., *H. leucopus* Pers., and *H. macropus* (Pers.) P. Karst have also been reported to be edible ascomycetous species which are consumed in fresh forms (Kaul 1971, 1978; Kumar and Sharma 2011; Dorjey et al. 2013). Although many species of *Helvella* and *Gyromitra* are edible (Kumar and Sharma 2011), there are also reports of gastrointestinal problems caused by some of their species. Many people are not aware about the edibility of

Gyromita sp. and consider it as poisonous (Chauhan et al. 2014). *G. esculenta* (Pers.) Fr. is reported to be deadly poisonous when eaten raw. Like *Helvella crispa*, it is also known as "Muh" and is eaten after boiling and then cooking. Water left after boiling is thrown away (Sagar et al. 2017).

Cauliflower Fungi *Sparassis* the cauliflower mushroom is the most cherished non-lamellate mushroom in Indian Himalayan region (Lakhanpal 1994; Kumar and Sharma 2011; Chauhan et al. 2014; Sagar et al. 2017). People here call it "Ban Gobhi" or "goat's meat" because it tastes like that of "Bhed Shairee," "Rao Gaub," or "Rao Gabur." Two common species found in these areas are *S. crispa* and *S. radicata*. Their cauliflower-like fruit body weighs up to 5–7 kg (Fig. 20.2a). Collection of *Sparassis* in every monsoon season is a common practice performed by the people here. The place where *Sparassis* occurs once is marked by the people and subsequently covered with grasses or bushes so as to hide the place. It can be found in coniferous forest commonly at the base of living *Cedrus deodara* (Roxb. ex D. Don) Loudon trees or on the stumps. Their fruit bodies are also preserved for future use. These are split into small pieces and dried in sun. About 2–3 days old fruit bodies are collected before they start decomposing. The fresh carpophores are washed thoroughly, chopped into small pieces, and cooked. The dried carpophores are soaked in water before use and they swell up almost to their original size. When plenty, these mushrooms find their place in local markets and are sold in fresh form at Rs 80–100 per kg.

Morels *Morchella* is an ascomycetous genus, many of whose species, viz., *Morchella angusticeps* Peck, *M. conica* Pers., *M. esculenta* (L.) Pers., *M. elata* (L.) Pers., *M. crassipes* (Vent.) Pers., *M. hybrida* (Sowerby) Pers., *M. rotunda* (Pers.) Boud., *M. semilibera* DC, and *M. tomentosa* M. Kuo, are found in the forests of Himalayas (Cooke 1879; Tilak and Rao 1968; Kaul 1975; Waraitch 1976; Kaul 1978; Kotwal et al. 2014). It is locally called as "Guchhi," "Gopal," "Jamoo," "Thunthoo," "Shiame," or "Cheon." Many of its species such as *M. esculenta*, *M. conica*, and *M. angusticeps* occur in mycorrhizal association with some herbaceous plants like strawberry, ferns, and grasses (Fig. 20.2b). Species of *Morchella* are cherished very much for making cuisine (Sohi et al. 1965; Lakhanpal 1994). Wild species are collected by local people and sold in local markets or to the traders at a high rate of about Rs. 5000–12,000 per kg (Chauhan et al. 2014). *Verpa conica* (O.F. Müll.) Sw. is a common adulterant mushroom found in the morel trade (Kumar et al. 2014). Edible morels are consumed by Himalayan people in various forms. It is cooked with rice and other vegetables and considered as highly nutritious like meat or fish. People of Bhotiya tribes of Central Himalayas use its decoction by boiling it in water, while local inhabitants of Kullu district (H.P.) are reported to boil it with milk (Prasad et al. 2002). In Sirmaur district, the fresh fruit bodies are first washed thoroughly with lukewarm water, and then its chopped pieces are cooked like other usual dry or soupy vegetables. Onions, tomatoes, salts, and spices are also used to add taste to soups and gravies due to its unique flavor.

Fig. 20.2 Non-lamellate edible wild mushrooms of NW Himalayas. (**a**) *Sparassis crispa*. (**b**) *Morchella esculenta*. (**c**) *Boletus edulis*. (**d**) *Auricularia auricula*. (**e**) *Rhizopogon luteolus*. (**f**) *Geopora arenicola*

Boletes Some of the boletes species such as *Boletus edulis* Bull., *B. hoarkii* T.N. Lakh. & R. Sharma, *B. granulatus* L., and *B. luridus* Schaeff. are also mushrooms of choice for consumption. Generally, these are found in mycorrhizal association with *Cedrus deodara. Boletus edulis* can be found sometimes associated with *Picea smithiana* Boiss. and *Abies pindrow* (Lakhanpal 1994; Kumar and Sharma 2011). In J&K, the edible boletes are named variously in local dialects. In Bhadarwahi local people call them "Dailoo" (fungus that easily shreds into fragments). Other local names in Bhadarwahi and Gaddaishi dialect are "Bhutol" and "Bhutoo" (consumed after roasting). *B. edulis* is one of the highly priced (Fig. 20.2c) commercial mushrooms (Hall et al. 1998a, b) which is known to have both culinary and medicinal value (Daba and Ezeronye 2003; Zheng et al. 2007). *Octaviania densa* (Rodway) G. Cunn. is another truffle-like, dark brown, fleshy bolete with dry peridium which grows subterranean or partly exposed in mycorrhizal association with *C. deodara* in the months of March to May. This is consumed both raw and after cooking. Raw sporophores are buried in burnt hot ash for roasting just like potatoes and then consumed after peeling off the peridium (Lakhanpal 1994; Kumar and Sharma 2011). *Suillus sibiricus* (Singer) Singer is another edible bolete, which is locally known as "Hotops" in Kamru area of Kinnaur District in Himachal Pradesh. It is used for cooking dry vegetable by the inhabitants of this area (Sagar et al. 2017).

Polypore Fungi An edible lignolytic macromycete, *Laetiporus sulphureus* var. *himalayansis* R. Yangdol, S. Kumar & Y.P Sharma, is one of the few polypores that are consumed in Trans-Himalayan region in Jammu and Kashmir (Yangdol et al. 2014). This fungus is regarded as a herbal medicine and is reported to contain compounds of therapeutic value (Zjawiony, 2004). In Bhoti language of Ladakh, this mushroom is referred to as "Chasha" (meaning chicken, because of its meaty chicken-like texture and taste). The robust fruiting bodies of this macrofungus are routinely collected during monsoon and consumed as a vegetable substitute. The sporophores are rinsed in water, sliced, and then cooked in combination with chopped onion, tomato, garlic, and the spices like turmeric, cumin, red chili powder, coriander, etc. or mixed with other vegetables and even consumed as mixed vegetable mushroom soup.

Jelly Fungi *Auricularia* is a genus belonging to the group of jelly fungi which are widely eaten by the people. It is represented by eight species worldwide (Kirk et al. 2008) that are characterized by cup-shaped gelatinous carpophores (Fig. 20.2d). Many of its species are consumed in different states of India (Sarma et al. 2010). *A. auricula-judae* (Bull.) J. Schröt. and *A. polytricha* (Mont.) Sacc. are the edible species generally found on living or dead woods of *Grevillea robusta* A.Cunn. ex R.Br., *Bauhinia malabarica* Roxb., *Delonix regia* (Bojer) Raf., and *Quercus leucotrichophora.* Locally these are called as "Muro" in Dehradun (Uttarakhand) and eaten in both fresh and revived dried forms by frying with onions (Semwal et al. 2014). *Auricularia polytricha* is known for its good proximate composition and plenty of minerals (Manjunathan et al. 2011).

Truffles Truffles (*Tuber* spp.) are another group of subterranean fungi found under the ground. In ancient times Greek and Romans used to collect these mushrooms with the help of dogs and pigs who could smell and locate them. Many of the wild truffles are being consumed all over the world and are of good economic utility. *Tuber indicum* Cooke & Massee is a preferred choice for food at a commercial scale in China (Mortimer et al. 2012). In the Indian Himalayan region, locally consumed wild tuber species are *T. cibarium* With. in Kangra (H.P.) and Kashmir and *Melanogaster durissimus* Cooke in Kangra, H.P. (Cooke 1879; Chopra and Chopra 1955; Das 2001).

False Truffles Appearing superficially like a potato and with variable size and shape, species of *Rhizopogon* (Fig. 20.2e), locally called as "Haildu" or "Dudh Kutt" (appearing like milky white after cutting), are the group of false truffles that are even eaten uncooked after cleaning and washing. The commonly gathered species of *Rhizopogon* Fr. from coniferous forests of NW Himalaya in Jammu & Kashmir include *Rhizopogon guzmanii* var. *macrospora* Kumar and Sharma, *R. luteolus* Fr., *R. luteolus* var. *Multiguttulata* Kumar and Sharma, *R. sublateritius* A.H. Sm., and *R. vulgaris* (Vittad.) M. Lange (Beig et al. 2011; Kumar and Sharma 2011, 2018). In Kamru area of Kinnaur District of Himachal Pradesh, *Rhizopogon* is reported to grow closely in association with *Cedrus deodara* and is locally known as "Mikalt." It is being cooked as dry vegetable like other mushrooms (Sagar et al. 2017).

Apothecial Fungi Likewise, *Geopora arenicola* (Lév.) Kers is also among the most delicious wild edible mushrooms (Fig. 20.2f). In different parts of J&K, it is known by various local names such as "Kundi" (Bhadarwahi and Gaddaishi), "Kutch" (Kishtwari), "Gav Padur" or "Khuduz" (Kashmiri), and "Kann Kutch" in Kishtwari and Kashmiri parlance (Kumar and Sharma 2011). For long-term preservation, the apothecia are washed and then sun-dried, salted, and laced with turmeric powder (Kumar and Sharma 2011).

20.5 Lamellate and Gasteromycetoid Edible Mushrooms

Agaricoid Mushrooms *Agaricus campestris* L., *A. arvensis* Schaeff., and *A. bitorquis* (Quél.) Sacc. are very common terrestrial, lamellate grassland mushrooms (Fig. 20.3a, b), found at lower altitudinal range of outer Himalayan belt during rainy season from July to September and consumed by the local people in several locations of Uttarakhand, Himachal Pradesh, and Jammu and Kashmir (Watling and Gregory 1980; Kumar and Sharma 2011).

Fig. 20.3 Agaricoid and Coprinoid wild edible mushrooms of NW Himalayas. (**a**) *Agaricus campestris*. (**b**) *A. bitorquis*. (**c**) *Coprinus comatus*. (**d**) *Coprinopsis atramentarius*

Coprinoid Mushrooms *Coprinus comatus* (O.F. Müll.) Pers. is one of the lamellate edible mushrooms. It is a deliquescent mushroom characterized by cylindrical to oval pileus at young stages and campanulate to conical with uplifted margins at maturity, a moveable annulus on the stipe and a bulbous base (Fig. 20.3c). It is collected and consumed by the local people of NW Himalaya as vegetable (Singh et al. 2017). It has been documented from Uttarakhand (Vishwakarma et al. 2012) and Jammu and Kashmir (Kaul and Kachroo 1974; Watling and Gregory 1980; Abraham et al. 1984, Kumar and Sharma 2009). There are a number of reports about its edibility by various investigators (Bose and Bose 1940; Purkayastha and Chandra 1985; Arora 1986; Atri et al. 2010). Few other forms such as *C. micaceus* (Bull.) Fr. and *Coprinopsis atramentarius* (Bull.) Fr. have also been reported to be edible from Jammu and Kashmir (Fig. 20.3d).

Lentinoid Mushrooms These are wood-decaying mushrooms which are characterized by xeromorphic habit, decurrent lamellae, and homoiomerous context having dimitic hyphal system (Fig. 20.4a). In recent times, a lot of work on the edibility and domestication of some of these mushrooms has been undertaken in Nigeria because of which they are reported to have assumed greater importance in the dietary preferences of both the rural and urban dwellers in the African subcontinent (Ogundana and Fagade 1982; Adejumo and Awosanya 2005; Nwanze et al. 2006). From India, Gulati et al. (2011) evaluated five edible species of *Lentinus*, namely, *L. sajor-caju* (Fr.) Fr., *L. connatus* Berk., *L. torulosus* (Pers.) Lloyd, *L. cladopus* Lév., and *L. squarrosulus* Mont., for their proximate nutritional composition. Protein content was found to be maximum in *L. torulosus* (2.45%), while lowest amount (0.48%) of crude fat was present in *L. connatus*. As far as crude fibers are concerned, maximum percentage was documented in *L. sajor-caju*. In comparison with all other species, *L. squarrosulus* contained maximum percentage of ash (2.21%). Among minerals, Ca and Mg were present in maximum proportion in *L. connatus*, while Zn and Na were present in maximum amount in case of *L. squarrosulus* in comparison to all other evaluated species. Copper (Cu) was in maximum amount in *L. torulosus*, while *L. cladopus* contained K in maximum amount among the evaluated species. Sharma and Atri (2014) also evaluated these species for their nutraceutical composition. In all the evaluated species, sucrose was found to be predominantly present in comparison to glucose and xylose. Among the fatty acids, saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), and polyunsaturated fatty acids (PUFA) were prominently documented. Major antioxidants recorded were ascorbic acid, β-carotene, lycopene, and phenolic compounds, while among amino acids, aspartic acid, arginine, alanine, proline, and tyrosine were documented in variable proportions. While evaluating these species for the presence of alkaloids, Sharma et al. (2013) found them in the range of 0.52 to 0.89 % with maximum amount in *L. cladopus*. Purkayastha and Chandra (1985) also documented the edibility of *Lentinus sajor-caju* from Tamil Nadu and that of *L. subnudus* from West Bengal and Orissa. Because of their culinary importance, domestication experiments were successfully done on three of these species, namely, *L. squarrosulus* (Atri et al. 2018), *L. connatus* (Atri et al. 2011), and *L. cladopus* (Atri and Lata 2013). In the North Western part of India, these are being collected for personal consumption from their natural habitats when they are young and soft in consistency.

Pleurotoid Mushrooms Among pleurotoid mushrooms, *Pleurotus* is the most commonly consumed edible mushroom. It is also referred as "Dhingri". There are a number of species of this genus including *P. citrinopileatus* Singer, *P. pulmonarius* (Fr.) Quél., *P. sapidus* Quél. (Fig. 20.4b), *P. sajor-caju* (Fr.) Singer, *P. salignus* (Pers.) P. Kumm., and *P. ostreatus* (Jacq.) P. Kumm., which are collected from the forested areas in the tropical and subtropical regions as well as Kashmir Province (Kaul and Kachroo 1974) of the outer Himalayan belt for culinary purposes

Fig. 20.4 Lamellate edible wild mushrooms of NW Himalayas. (**a**) *Lentinus squarrosulus*. (**b**) *Pleurotus sapidus*. (**c**) *Macrolepiota procera*. (**d**) *Termitomyces heimii*. (**e**) *Amanita vaginata*. (**f**) *Volvariella volvacea*

(Lakhanpal 1994; Atri et al. 2012b, 2013). Another species *P. platypus* Sacc. has been reported to be edible from Kashmir (Abraham and Kaul 1990). In Sirmauri dialect, its species coming up on dried logs of *Euphorbia royleana* Boiss. are referred as "Suru Koir." Here "Suru" refers to the host plant and "Koir" refers to the mushroom. In Doda district of J&K, edible *Pleurotus squarrosulus* (Mont.) Singer is denoted by many vernaculars such as "Saroori," "Srij," and "Sirer" (Kumar and Sharma, 2011). While investigating the nutritional and nutraceutical profile of the wild samples of *P. pulmonarius*, *P. sapidus*, and *P. sajor-caju*, Atri et al. (2013) documented carbohydrate proportion to range from 88.38 to 86.73%, dietary fibers from 2.98 to 2.76%, protein from 1.66 to 0.98%, ash content from 1.91 to 1.03%, and crude fats from 0.79 to 0.62%. Both macro- and microminerals including Ca, Mg, Na, K, Cu, Zn, and Fe were found in appreciable proportion. None of the heavy metals including Pb, Hg, As, Sb, and Ag were documented from these wild species evaluated. Among the sugars, sucrose was more in comparison to glucose and xylose. With respect to fatty acids, MUFAs were documented in higher proportion in comparison to SFAs. In terms of antioxidant composition, all the species contained ascorbic acid, phenols, β-carotene, and lycopene. Yet another new variety of *Pleurotus*, *P. pulmonarius* var. *indicus* Sapan, Atri & Gulati, was described from Palampur in Himachal Pradesh, which was quite rich in its nutritional and nutraceutical credentials like its type variety (Sharma et al. 2014). In view of the pharmaceutical relevance of alkaloids, these mushrooms when evaluated were found to contain alkaloids in the range of 0.62–0.74% (Sharma et al. 2013). Variable proportion of different amino acids including aspartic acid, arginine, alanine, proline, and tyrosine were documented in all these evaluated mushrooms (Atri et al. 2012b).

Macrolepiotoid Mushrooms Macrolepiotoid mushrooms, namely, *Macrolepiota procera* (Scop.) Singer, *M. dolichaula* (Berk. & Broome) Pegler & R.W. Rayner, and *M. rhacodes* (Vittad.) Singer, are well-known for their edibility (Fig. 20.4c). They are reported to be traditionally consumed in Indian subcontinent (Purkayastha and Chandra 1976, Kumar and Sharma 2009; Atri et al. 2010). These mushrooms contain 16.45–19.95% protein, 2.9–3.4% fat, 2.5–5.1% crude fibers, 56.2–68.19% carbohydrates, and 1.93–7.3% ash content. They are also rich in minerals and contain Ca, Mg, Cu, Fe, Mn, Zn, Se, Hg, Cd, As, etc. From the nutraceutical point of view, 16–81 mg/g of phenols, 1.36-1.76 mg/g of flavonoids, 0.048–0.103 mg/g of alkaloids, 0.12–0.29 μg/g of β-carotene, and 0.05–0.12 μg/g of lycopenes were documented from these mushrooms (Kumari and Atri 2014). The presence of such natural nutrients and nutraceutics make these commodities suitable for consumption by the modern calorie-conscious society. Similarly, the significant levels of proteins, fats, carbohydrates, fibers, and some nutraceutics in their sporophores make macrolepiotoid mushrooms of considerable value from culinary point of view. In Sirmauri dialect in Himachal Pradesh, these mushrooms are known as "Kandi Koir." Here, "Kandi" refers to annulus and "Koir" refers to mushroom.

Termitophilous Mushrooms *Termitomyces* is a basidiomycetous mushroom genus. Some of its species which can be commonly encountered in tropical and subtropical region of NW Himalayas are *T. microcarpus* (Berk. & Broome) R. Heim, *T. heimii* Natarajan, *T. mammiformis* R. Heim, *T. schimperi* (Pat.) R. Heim, etc. (Fig. 20.4d). Their unique flavor and exotic taste make them superior to other edible mushrooms including *Agaricus bisporus* (J.E. Lange) Imbach, *Pleurotus* sp., *Volvariella volvacea* (Bull.) Singer, etc. Except for *T. microcarpus* and *T. medius* R. Heim & Grassé, most of its species are thick-fleshed, with fibrous soft texture comparable to egg, meat, etc. (Pegler and Piearce 1980; Atri et al. 2005).

During monsoon season, besides collection for personal consumption, some of the species of *T. heimii*, *T. mammiformis*, *T. striatus* R. Heim & Grassé, *T. clypeatus* R. Heim, and *T. eurrhizus* (Berk.) R. Heim are collected in bulk from the wild and are sold by vegetable vendors in some parts of North India at Rs. 50–60 per kg (Atri et al. 2005; Kumar and Sharma 2009, 2011). Atri et al. (2012a) evaluated many species including *T. microcarpus*, *T. medius*, *T. heimii*, and *T. mammiformis* for nutritional and nutraceutical constituents. Protein content was found to be ranging from 12.95 to 46.20 %, carbohydrates 33.3 to 60.27%, fibers 2.5 to 8%, crude fat 1.65 to 3.3%, and ash 5 to 15.6%, and the energy value was found in the range of 306.50–351.80 KJ/100g. In case of nutraceutical components, it was found to contain phenol (15.04–25.85 mg/g), flavonoids (1.38–2.02 mg/g), ascorbic acid (0.02–0.18 μg/g), and carotenoids (0.11–50 μg/g). Kumari and Atri (2012) documented appreciable quantities of alkaloids ranging from 0.046 to 0.77 mg/g from different species of *Termitomyces*. These deep pseudorhizal mushrooms are commonly referred to as "JoruKoir" in Sirmauri dialect. *Termitomyces* species along with its long pseudorhiza are collected in both opened and unopened stages, washed with lukewarm water, and then cooked as a dry or mixed vegetable. Some people prefer boiling the sporophores before cooking due to their cartilaginous texture. These are also prepared as mushroom curry and a vegetable with good amount of soup. It takes about 20 min tes to cook dishes of these mushrooms. *T. microcarpus* is commonly referred as "Bhat Koir" in Sirmauri dialect in Himachal Pradesh. Technically "Bhat" refers to cooked rice, and "Koir" refers to mushroom, since these mushrooms are cooked with rice.

Amanitoid Mushrooms Amanitoid mushrooms are peculiar in having both annulus and volva in most of their species. *A. hemibapha* (Berk.& Broome) Sacc. is an edible wild species found on the forest floor in ectomycorrhizal association with a number of tree species found in Himalayas at varying altitudes such as *Shorea robusta*, *Picea smithiana*, *Lyonia ovalifolia*, *Abies pindrow*, and *Cedrus deodara.* It is locally consumed by people of North West Himalaya. This species has been reported from Uttarakhand and Himachal Pradesh at various elevations ranging from 650 m to 2300 m (Upadhyay et al. 2008; Kaur et al. 2008; Semwal et al. 2014). Similarly *A. chepangiana* Tulloss & Bhandary, *A. caesarea* (Scop.) Pers., and *A. vaginata* (Bull.) Lam. (Fig. 20.4e) have also been documented as edible wild species of North West Himalaya (Lakhanpal 2002; Vishwakarma et al. 2012; Semwal

et al. 2014) which are used for culinary purposes by inhabitants of the area. *A. caesarea* is known to be used after boiling well (Lakhanpal 2002). *A. vaginata* is called as "Ghia Koir" in Sirmauri dialect due to its shinning or glistening pileus surface. It is commonly found wild in pine and oak forests in Sirmaur. People collect its opened fruit bodies during monsoon season and cook it as a dry vegetable. Fresh fruit bodies are washed with lukewarm water, chopped into pieces, and then shallow-fried in oil/ghee with chopped onions, tomatoes, garlic, salt, and other common spices as per one's taste. It is also cooked by mixing with other mushrooms and vegetables. It takes about 15–20 min to cook this mushroom.

Volvarioid Mushrooms Species of volvarioid mushrooms are characterized by medium- to large-sized carpophores, pink-colored basidiospores, and a bulbous base with or without volva (Fig. 20.4f). There are reports of consumption of wild species of *V. volvacea* (Bull.) Singer as food by local inhabitants of tropical and subtropical parts of outer Himalaya as well as other parts of India such as Punjab and West Bengal (Atri et al. 2010; Dutta and Acharya 2014). *V. volvacea* is also known to have antimicrobial and anticancer properties (Wu et al. 2011; Giri et al. 2012).

Russuloid Mushrooms Russulaceous mushrooms, being mycorrhizal with a number of angiospermic and gymnospermic plants, play an important role in nurturing the associated plants with the required nutrients including minerals. They are vividly colored and are characterized by the presence of heteromerous context, variously ornamented basidiospores, and absence of clamp connections in the mycelium. Some of these mushrooms including *Russula aeruginea* Lindblad, *R. aurea* Pers., *R. delica* Fr., *R. cyanoxantha* (Schaeff.) Fr., *R. lutea* (Huds.) Gray, *R. virescens* (Schaeff.) Fr., *R. brevipes* Peck, *R. heterophylla* (Fr.) Fr., *Lactarius deliciosus* (L.) Gray, *L. sanguifluus* (Paulet) Fr., *Lactifluus piperatus* (L.) Pers., *L. volemus* (Fr.:Fr.) Kuntze, etc. (Fig. 20.5a–f) are highly delicious and are collected regularly during monsoon season for personal consumption in North western part of India, Europe, other Asian countries, and North America (Hesler and Smith 1979, Watling and Gregory 1980; Beig et al. 2011; Pala et al. 2012; Singer 1986, Sharma et al. 2017). There are reports of their marketing in the European markets (Singer 1986). Enormous quantities of *Lactarius deliciosus*, *L. sanguifluus*, *L. resimus* (Fr.) Fr., and *L. scrobiculatus* (Scop.) Fr. are reported to fetch very high price in the markets of Russia and Barcelona. So far we have not come across any instance of their marketing in India. Their complete nutritional and nutriceutical profile was evaluated from the wild samples collected from different localities of North West Himalaya by Sharma et al. (2017). Sagar et al. (2017) documented the edibility of *L. deliciosus* and *L. sanguifluus* from Kamru region of Kinnaur District in H.P. In this area these mushrooms are locally referred as "Santri Chattri" and "Pili Chattri," respectively. Nutritional analysis confirmed the presence of substantial amount of protein which ranged from 19.84 to 37.77% and carbohydrates from 40.81 to 63.24%, fats were

Fig. 20.5 Russuloid wild edible mushrooms of NW Himalayas. (**a**) *Russula cyanoxantha*. (**b**) *R. brevipes*. (**c**) *R. virescens*. (**d**) *Lactifluus volemus*. (**e**) *Lactarius sanguifluus*. (**f**) *L. deliciosus*

quite low and ranged from 1.7 to 5.44%, and the ash content was 6.17–16.43%. The energy value was also calculated which ranged from 253.84–287.40 kcal/100 g of the mushroom sample. Mannitol and trehalose were the main sugars in all the evaluated samples of these mushrooms. Among the nutraceutical components, phenolic content ranged from 1.78 to 17.55 mg/g, flavonoid content from 0.14 to 2.4 mg/g, ascorbic acid from 0.12 to 0.31 mg/g, β-carotene from 4.47 to 32.73 μg/g and the reducing power of the mushroom methanolic extract from 0.05 to 0.77. Slavic populations in Eastern Europe are reported to use the edible species of *Russula*, especially *R. cyanoxantha* for pickling or salting (Singer 1986). Among the edible species of *Russula*, Sagar et al. (2017) documented the edibility of *R. brevipes* from Kamru region of Kinnaur District, where it is locally referred as "Kaithno or Kaithmuh" and is cooked as mixed vegetable along with peas.

Gasteromycetoid Mushrooms *Podaxis*, a gasteromycetous fungus belonging to family *Agaricaceae*, is known by its ten species worldwide (Kirk et al. 2008). Its most common species *P. pistillaris* (L.) Fr. is consumed at various places on the globe and India itself (Morse 1933; Arora 1986; Batra 1983; Mridu and Atri 2015). It is characterized by white fleshy gleba which has various nutritional and medicinal values (Fig. 20.6a). *P. pistillaris* has been reported to be used for the treatment of inflammation and skin disease (Gupta and Singh 1991; Mao 2000). It is also reported to exhibit antimicrobial activity (Panwar and Purohit 2002). In the tropical and subtropical regions of Himalayan belt, local people collect it as vegetable for personal consumption after cooking. Young fresh sporocarps of this mushroom are collected and washed thoroughly, chopped, and fried in oil/ghee and other usual ingredients.

Lycoperdon Tourn.ex L. is another gasteromycetous mushroom genus which is a common representative of "puff balls." It is locally called as "Lalari" or "Lalrishal" by the people in Kinnaur district (H.P.), "Dimla Koir" by the inhabitants of Sirmaur (H.P.), and "Ophands" in Bhadarwah tehsil (J&K). These are normally collected in the months of July-September and consumed fresh as well as dried as a vegetable (Fig. 20.6b). People first boil the fresh fruit bodies in water and then cook it with usual ingredients just like other mushrooms. Surplus collection of this mushroom is preserved by sun-drying for off-season consumption (Chauhan et al. 2014). Wild edible species of *Lycoperdon* reported from Himalayas include *L. curtisii* Berk. (Mussoorie, Uttarakhand), *L. perlatum* Pers. (Dalhousie, H.P.), *L. muscorum* Morgan (Chail, H.P.), *L. pyriforme* Pers. (Kullu, H.P.; Sonamarg, J & K), *L. rimulatum* Peck. (Shimla, H.P.) and *L. umbrinum* Pers. (Kullu, H.P.; Sonamarg, J & K) (Ahmad 1942, Sohi et al. 1964, Gupta et al. 1974, Bilgrami et al. 1979). Some of its species including *L. perlatum* Pers., *L. pusillum* Batsch, and *L. giganteum* Batsch are also known to have antimicrobial and antioxidant properties (Jonathan and Fasidi 2003; Ramesh and Pattar 2010). Two species of *Calvatia*, namely, *C. bovista* (L.) Pers. and *C. lycoperdoides*, which are commonly referred to as "Khucoon" or "Hand" in Ladakh are also collected for personal consumption by the local people (Dorjey et al. 2016). *Bovista pusilla* (Batsch) Pers. is a representative of true

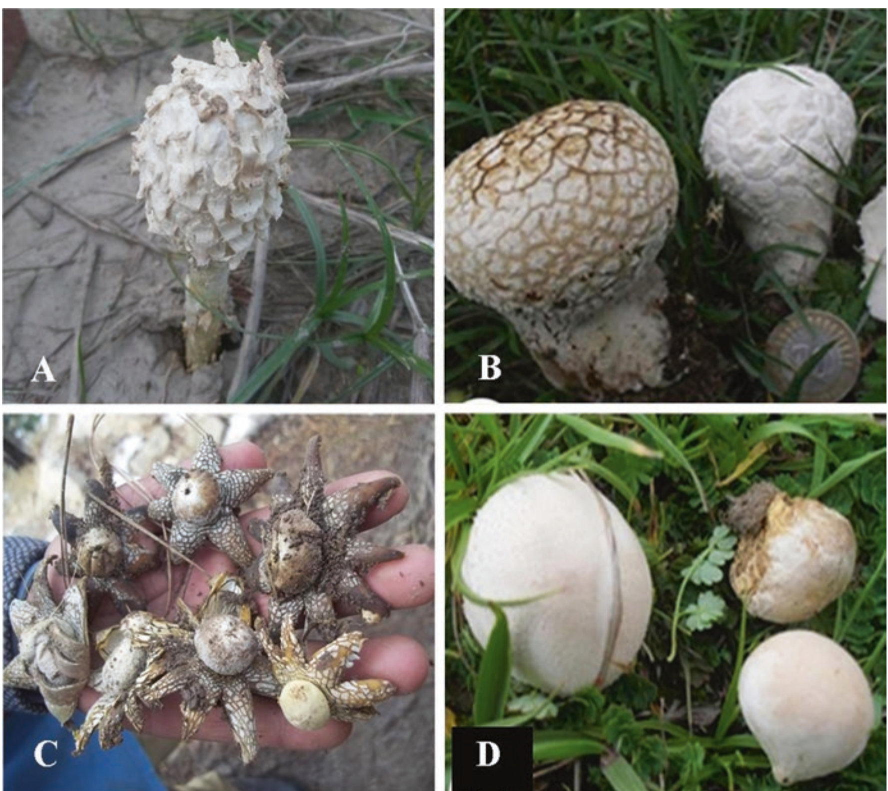

Fig. 20.6 Gasteromycetoid wild edible mushrooms of NW Himalayas. (**a**) *Podaxis pistillaris*. (**b**) *Calvatia bovista*. (**c**) *Astraeus hygrometricus*. (**d**) *Bovista pusilla*

puffball which is an important edible mushroom in North West Himalayan region. When young, it appears white from inside just like *Lycoperdon*. (Fig. 20.6d). *Astraeus hygrometricus* (Pers.) Morgan, commonly referred to as hygroscopic earth star, also looks like puff ball when young and unopened (Fig. 20.6c). These are being collected by locals in J&K for personal consumption. These are being marketed and sold in Thailand markets. Pavithra et al. (2015) documented its edibility from Western Ghats in India.

20.6 Preparation of Mushroom Recipies and Caution

Mushrooms are part of one of the unconventional sources of food which of late have started finding prominent place in the Indian menu. Mushrooms constitute purely vegetarian diet, and unlike most vegetables, mushrooms retain their shape and do not dissolve with the gravy while cooking. A single steam pressure is reported to be enough to cook and retain their nutrients (Mehta 1990).

For consumption purpose mushrooms should be gathered when they are fresh and young and not infested with maggots. Older sporophores should be avoided especially in case of *Agaricus*, *Coprinus*, *Podaxis*, *Lycoperdon*, *Calvatia*, etc. In comparison *Cantharellus* has good shelf life and can be used to make delightful dishes. Even edible species of *Lentinus* also have good shelf life. Among the russulaceous mushrooms, *Russula cyanoxantha*, *R. virescens*, and *Lactarius deliciosus* are most sought after mushrooms because of their crunchy texture and excellent flavor. Among the ascomycetous mushrooms, *Morchella* and *Tuber* also fall in the same category as far as the shelf life, edibility, and taste are concerned. Species of *Pleurotus*, *Macrolepiota*, *Amanita*, *Volvariella*, *Termitomyces*, etc. are some of the fleshy mushrooms which require immediate attention after collection to prevent their microbial decomposition and enzymatic browning so as to retain their consistency and texture.

Before proceeding for cooking, mushroom carpophores should be thoroughly washed or properly wiped out with damp cloth. Bottom portion of the stipe should be discarded before slicing the carpophores into small pieces. During cooking mushrooms can be blended with any other vegetable, eggs, or meat as per individual taste so as to make a variety of dishes including sandwich, omelette, soup, salad, pizza, vegetable stuffings, mushroom mutton, mushroom rice, mushroom pulao, mushroom tikka, and pickles (Fig. 20.7e, f). Information about the composition of specific recipes can be obtained from the technical bulletin number-3 published by National Centre for Mushroom Research and Training (now Directorate of Mushroom Research, ICAR), Chambaghat, Solan (Mehta, 1990), and from the book entitled *The Ultimate Mushroom Book: The Complete Guide to Mushrooms* by Jordan and Wheeler (1995).

Atri et al. (2010) enumerated some points which can be of help to broadly segregate inedible mushrooms from the edible and preferred ones. Random hunting for personal consumption in an indiscriminate manner without much knowledge about the edibility can be a dangerous preposition which may result even in the loss of one's life. Therefore, it is always advisable not to eat a mushroom unless it is correctly identified as edible by some experienced mushroom forager whose judgment you have good reason to trust. As stated by Trudell and Ammirati (2009), "Remember that no meal is worth ending your life, when in doubt, throw it out." Some of the species including *Chlorophyllum molybdites* and *Helvella crispa*, although edible, may result in gastrointestinal disorders. As stated by Lincoff and Mitchell (1997), "No Rule is the only Rule" with regard to the edibility of mushrooms. They should be learnt and tasted species by species for eating purposes. Normally mushrooms which are mild tasting with agreeable odor and are eaten by rodents, squirrels, and monkeys in the forests are considered as safe, while the species with both annulus and volva (*Amanita*) and those tasting acrid and having obnoxious smell are considered as unsafe from culinary point of view.

Fig. 20.7 Sociobiology of edible wild mushrooms of NW Himalayas. (**a**–**c**) Local people collecting wild edible mushrooms for consumption. (**d**) Interacting with local people to procure information about mushrooms. (**e**) A dish made a wild mushroom species. (**f**) A dish made from Laetiporus sulphureus

20.7 Conclusions

The research studies indicate that several surveys have been conducted on the diversity of mushrooms of the North West Himalaya, yet very little is known on their nutritional, nutraceutical, and therapeutic status. In view of the increasing commercialization, more studies on the wild edible mushrooms and their ethnomycology in the Himalayas are called for. Further, it will be worthwhile to gather different views of the local populace about the value of mushrooms, which would pave a way for the introduction of some known wild edible mushrooms in the diet of rural population. Several mushroom species have been pointed out as sources of bioactive compounds, in addition to their important nutritional value. They possess low fat content, because of which these can be used in low-calorie diets, from nutritional point of view. Some formulations could be used as antioxidants to prevent oxidative stress, and thus, these can play an important role against aging process. Future studies into the biochemical analysis and mechanisms of action of mushroom extracts will help us to further delineate the interesting roles and properties of various mushroom bioactive components in the prevention and treatment of some degenerative diseases. Because of their nutritional and nutraceutical relevance, these mushrooms need to be domesticated by adopting simple and appropriate low-cost technology. This becomes even more relevant when Food and Agricultural Organization has recommended the use of edible mushrooms as food supplement for protein-deficient populations of developing and underdeveloped countries.

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21 Methanogens for Human Welfare: More Boon Than Bane

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Abstract

Microorganisms that produce methane as an end product of their energy-generating metabolism are known as methanogens. They represent phylum Euryarchaeota and are one of the most diverse groups of archaea. The ability of methanogens to utilize carbon dioxide and other by-products of bacterial metabolism as a 'C' and 'E' source and eventually convert them into methane has become the focus of recent research. Methanogens make significant contribution to global warming through emission of methane, the greenhouse gas. Biogenic methane in the form of methane hydrate trapped in subsurface sediments amounts to massive deposits of methane. Methane emissions from such deposits as a consequence of tectonic shifts can contribute to global warming through creation of ozone holes. Methane hydrates on the other hand can also serve as an untapped source of energy. Methane, if recovered, can be used as fuel, for heating, electricity/energy production and also for the synthesis of valuable chemicals. Methanogenic waste treatment of high-strength industrial waste has made the effluent treatment a cost-efficient process rather than a cost-intensive one. Although emissions of methane have adverse impact on the environment, the desirable use of methanogens for reducing environmental pollution, renewable energy generation and the synthesis of valuable chemicals has made methanogens in the environment a 'boon rather than bane'.

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Keywords
Methanogens · Archaea · Anaerobes · Extreme environment · Biogas production · Wastewater treatment · Global climate change

21.1 Introduction

Methanogens are archaea that are considered as a special microbial assemblage capable of producing methane as an end product of anoxic respiration. It has been observed that methanogens constitute a morphologically diverse group of microorganisms. Physiologically, they form a coherent group of obligate anaerobes and share a common metabolic capacity to produce methane. Methanogenesis was first discovered in 1776 by the Italian physicist Alessandro Volta who described 'combustible air' formed in the sediments of streams, bogs and lakes full of rotting organic material (Zinder 1993). Omelianski subsequently defined the microbial involvement in the generation of the methane gas (McInerney et al. 1979). Most of the methanogens known till date used $H_2 + CO_2$ as a substrate for their growth. In addition, some methanogens use volatile fatty acids (such as formic and acetic acids), dimethyl sulphides, alcohols (such as methanol, isopropanol, iso-butanol, cyclopentanol and ethanol) and amines such as methylamines as 'C' and 'E' sources which are metabolized leading to the formation of methane as an end product (Guerrero 2001). During the course of such metabolic conversion, methanogens obtain energy necessary for their growth. Excess energy is released by the cells in the form of methane gas (Fig. 21.1). Methanogens do not get energy for growth from any other cell process. Thus, conversion of substrate to methane is an obligate metabolic process for the methanogens to grow and survive (Ferry 1992). Certain *Clostridia* have also been reported to show methane production in minor quantities during growth. But this methane synthesis is not obligatory for energy generation. In fact, methane is a by-product of side reactions in their metabolism (Rimbault et al. 1988).

$$CO_2 + 4H_2 \rightarrow CH_4 + 2H_2O - \mathbf{135.1}\Delta G^{o\prime} \text{ kJ/mol } CH_4$$

$$\mathbf{4CO + 2H_2O \rightarrow CH_4 + 3CO_2 - 211}\ \Delta G^{o\prime} \text{ kJ/mol } CH_4$$

$$4HCOOH \rightarrow CH_4 + 3CO_2 + 2H_2O \Delta G^{o\prime} \ \mathbf{-119} \text{ kJ/mol } CH_4$$

$$4CH_3OH \rightarrow 3CH_4 + CO_2 + 2H_2O \text{ - } \mathbf{106}\ \Delta G^{o\prime} \text{ kJ/mol } CH_4$$

$$4CH_3NH_2 + 2H_2O \rightarrow 3CH_4 + CO_2 + 4NH_3 \ \mathbf{-76.7}\ \Delta G^{o\prime} \text{ kJ/mol } CH_4$$

$$4(CH_3)_2NH + 2H_2O \rightarrow 3CH_4 + CO_2 + 2NH_3 \mathbf{-74.8}\ \Delta G^{o\prime} \text{ kJ/mol } CH_4$$

$$2(CH_3)_3NH + 6H_2O \rightarrow 9CH_4 + 3CO_2 + 4NH_3 - \mathbf{75.8}\Delta G^{o\prime} \text{ kJ/mol } CH_4$$

$$2(CH_3)_2S + 2H_2O \rightarrow 3CH_4 + CO_2 + 2H_2S \mathbf{-52.1}\ \Delta G^{o\prime} \text{ kJ/mol } CH_4$$

$$CH_3COOH \rightarrow CH_4 + CO_2 \mathbf{-36.0}\ \Delta G^{o\prime} \text{ kJ/mol } CH_4$$

Fig. 21.1 Reactions involved in methane biosynthesis by methanogens. (Ferry 2012)

Methanogens can be classified into three categories based on their ability and mode of substrate utilization (Whitman et al. 2006). These are (1) hydrogenotrophic methanogens that have the ability to use CO_2 as the electron acceptor and either H_2, formate or alcohols as an electron donor. These methanogens reduce CO_2 to methane. Methanogens belonging to genera *Methanobacterium*, *Methanobrevibacter* and *Methanoculleus* are common examples of this group. (2) Methylotrophic methanogens constitute the second group, and they reduce methyl group to methane for their energy requirements. Trimethylamine, methanol and dimethylsulphide are the substrates for methanogens belonging to this group. In this process, the methyl group is directly reduced to methane, whereas the remaining is oxidized to CO_2. *Methanococcus* is the first methanogen reported from this group. (3) Acetoclastic methanogens constitute the third group which obtains energy during acetate catabolism. Members of *Methanosarcina* and *Methanospirillum* are the only examples of these methanogens. The acetoclastic methanogens reduce methyl group to methane and oxidize carboxyl group to CO_2 during catabolism of acetate. Bryant et al. (1967) reported close association between non-methanogen and methanogen for the first time. They resolved the so-called pure culture of *Methanobacillus omelianskii* growing on ethanol by producing methane and another nonmethanogen, called S organism, utilizing ethanol and producing HCO_3^- and H_2. Further, the methanogenic partner isolated in pure culture was *Methanobacterium* strain MoH which utilized H_2 and CO_2 to produce methane. Later on, Boone and Bryant (1980) reported *Syntrophobacter wolinii*-degrading propionate in close association with hydrogenotrophic methanogen. McInerney et al. (1981) described yet one more syntrophic association. They isolated *Syntrophomonas wolfei* utilizing butyrate in association with methanogen. H^+ produced by syntrophs was utilized by the methanogen. Utilization of H^+ by methanogens is essential for the growth of syntrophs as accumulation of H^+ is inhibitory to syntrophs. Also, lowered pH of the growth medium is not optimum for methanogen to grow.

In addition to fastidious requirement, methanogens also require low oxidation reduction potential and limited range of compounds as potential ‘C’ and ‘E’ source for their growth. Majority of the methanogens reported till date are hydrogenotrophic. During anaerobic decomposition of organic matter, many fermentative bacteria generate H_2 as one of the major end products along with CO_2. Thus, the methanogens act as a sink for hydrogen and facilitate microbial degradation in oxygen-limited environments. Acetate is another important end product of anaerobic decomposition. The acetoclastic methanogens are commonly observed in such kinds of environments where acetate is available in abundance (Demirel and Scherer 2008a, b). In comparison to H_2 and acetate, other methanogenic substrates are rarely produced in anaerobic environments. Methylamines, particularly trimethylamines, are associated with salt-rich environments. High concentration of betaine, a common osmolyte, has been reported in many organisms growing in saline and hypersaline environments (Watkins et al. 2014). Anaerobic decomposition of the biomass in these environments also induces anaerobic breakdown of betaine and choline resulting in production of methylamines mainly trimethylamines. Therefore, it is

not surprising to find that many of the methylotrophic methanogens reported are of marine origin (McGenity and Sorokin 2018).

Methanogenesis takes place over a broad temperature range. Rate of methane formation by methanogens is significantly reduced below 15 °C. Methanogens are reported from low-temperature environments to hyperthermophilic vents. Only one methanogen, *Methanogenium frigidum*, optimally grows at 15 °C (Franzmann et al. 1997). Methanogens grow well over temperature range of 20 to 45 °C which is same as that of most of the common habitats for methanogens which include animal intestines and rumen, most of the paddy fields, aquatic sediments, man-made anaerobic digesters and biogas reactors, etc. Methanogens such as *Methanothermobacter thermautotrophicus* are thermophiles with optimum temperature for growth between 50 and 80 °C. Members of this genus were obtained from marine sediments, hydrothermal vents, oil fields, anaerobic digesters, thermal springs (Wasserfallen et al. 2000; Guerrero 2001) and even from mud of the cattle pasture. Methanogens like *Methanocaldococcus* species with optimum temperature around 82–85 °C are hyperthermophiles (Bellack et al. 2011; Stewart et al. 2015). *Methanopyrus kandleri* is probably the only methanogen optimally growing at 98 °C, the highest optimum growth temperature reported for any of the methanogens (Kurr et al. 1991). Most of the thermophilic and hyperthermophilic methanogens are hydrogenotrophic, whereas only a few are acetoclastic or methylotrophic methanogens. Some methanogens do produce 'heat shock proteins' (Hebert et al. 1991), and some have ether-linked isoprenoid lipids which probably help their growth above 90 °C (Zinder 1993).

Methanogenesis occurs over a narrow range of pH, i.e. in the range of 6.5 to 8.0. Most of the methanogens fall in this group. There are moderately alkaliphilic methanogens such as *Methanobacterium thermoalcaliphilum*, *Methanocalculus alkaliphilus*, *Methanocalculus natrophilus*, *Methanosalsum zhilinae* and *Methanosalsum natrophilum* which show optimum growth at alkaline pH of 9.0 to 9.5 (Bräuer et al. 2006; Cadillo-Quiroz et al. 2014). Methanogenesis or growth of methanogens is rarely observed in acidic pH range of 5.0 to 6.0. One such example is of *Methanobrevibacter acididurans*, isolated from a sour anaerobic digester (Savant et al. 2002).

Methanogens are the most obligate anaerobes when compared with other archaea, bacteria or fungi. These archaea in pure culture only grow when the oxidation/reduction potential of the growth medium reaches near to −300 mV (Hungate 1969). Oxygen concentration in nutrient medium at such low redox potential is approximately 10^{-56} moles per litre. Hausinger et al. (1985) reported inhibition of reductive metabolism, particularly of coenzyme F_{420}, in *Methanobacterium thermoautotrophicus* upon exposure to air. In spite of such fastidious requirement for the absence of oxygen, during their growth and pre-reduced growth media, some methanogens have limited tolerance to oxygen exposure. Kiener and Leisinger (1983) have reported ~1% viability of *Methanococcus voltae* and *M. vannielii* after 10 h of exposure to air. They also reported that *Methanosarcina barkeri* could maintain viability even for 24 h. Their reports on oxygen sensitivity were for the pure culture growth experiments. Rastogi et al. (2008) showed that methanogens belonging to orders Methanobacteriales and Methanosarcinales were detected even in

20-month-old air-dried cattle waste. Ranade et al. (1979) demonstrated that methanogenic potential of dried cattle dung was retained even after the storage for 28 months. Such adaptation to air or oxygen could be attributed to low-level expression of superoxide dismutase in methanogens (Kirby et al. 1981). It is also likely that the energy or nutrients stored in the form of glycogen polymer and polyphosphate possibly help methanogens survive for long time in sludge of anaerobic digesters and also in dry cattle waste.

21.2 Phylogeny and Taxonomy of Methanogenic Archaea

Methanogenic archaea are lithotrophic in nature and get energy by the process of methanogenesis. They are obligate anaerobes, prokaryotic in nature and widely distributed in diverse ecological niches (Bintrim et al. 1997; Costa and Leigh 2014). They are mesophilic, thermophilic or psychrophilic in nature and represent a wide range of morphological, physiological and genetic variations. Jabłoński et al. (2015) have created a database of methanogenic archaea which contain information about all novel taxa of methanogens along with its physiological and biochemical features like range and optima of temperature, pH and salinity for the growth. Similar to other groups of bacteria and archaea, it has been found that only phenotypic information is not sufficient to identify the new taxa of methanogens. Authentic classification of methanogenic archaea needs study of phylogeny using 16S rRNA gene-based sequence similarity, membrane lipid composition, antigenic fingerprinting and data from genotyping. Thus, being prokaryotic in nature similar to bacteria, polyphasic approach and rules also apply for archaeal classification (Rosselló-Móra and Amann 2015; Zuo and Hao 2015) (Fig. 21.2).

Boone and Whitman (1988) proposed essential standards for description of new taxa of methanogens which was accepted by subcommittee for taxonomy of methanogenic bacteria. At the time of publication of the paper of Boone and Whitman (1988), the difference between bacterial and archaeal domain was not very clear-cut, and archaea was considered the part of domain bacteria due to its prokaryotic nature. Later in the late 1980s, it became clear that bacteria and archaea belong to separate domain in the three domains of the 'phylogenetic tree of life'. In light of the above statement, the term methanogenic bacteria is no more appropriate, and the article should be amended as the proposed minimal standard for taxonomy of methanogenic archaea with new inclusion and modifications. Unfortunately, after the publication of Boone and Whitman (1988) in the area of proposed minimal standard for the classification of methanogenic archaea came to light, we are still following the same guidelines. It is nearly 30 years back, and in the context of new technological developments and novel findings, the proposed minimal standard for taxonomical classification of methanogenic archaea needs revisit or revaluation. Being prokaryotic in nature, the phylogenetic, phenotypic and chemotaxonomic criteria for classification of bacteria and archaea follow almost similar guidelines with minor exception at taxa specific traits. In brief, according to minimal standard proposed for taxonomy of methanogenic bacteria (now methanogenic archaea) by

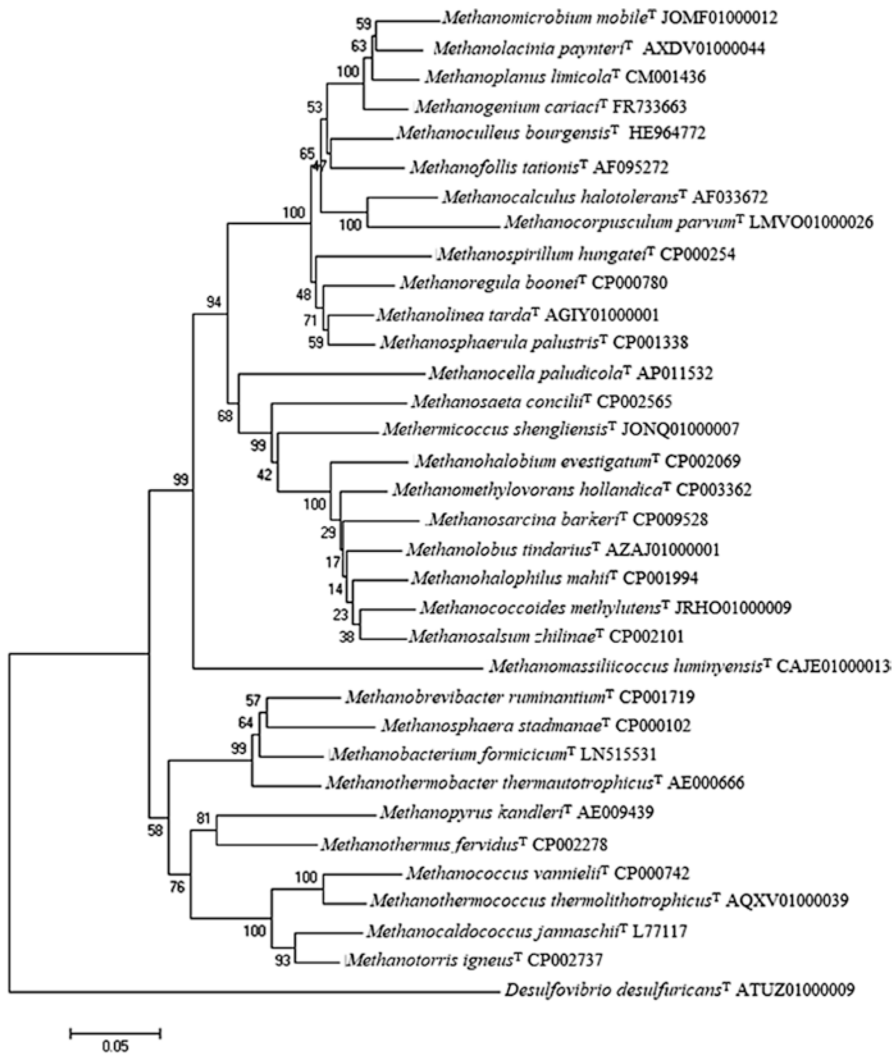

Fig. 21.2 Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing methanogens belonging to different genera. Bootstrap values based on 1000 replicates for the neighbour-joining are shown at the branch points. Bar 0.05 nucleotide substitutions per site

Boone and Whitman (1988), culture purity is the basic and prerequisite. Although, in case of methanogens, isolation and purification is a difficult process, it can be achieved using successive re-streaking on the surface of solid medium followed by single colony isolation re-suspension in respective media and serial dilution and

re-streaking. Unlike usual culture, it is a little bit tricky, but according to the nature of organism, solid medium agar plates, roll tube or agar slopes can be used for this purpose. Test for susceptibility to cellular lysis is another parameter and can be reported using different conditions. Due to lack of true peptidoglycan in archaeal cell wall, Gram staining is not a very important criterion of dissection, but it should be used to expand the resolution of comparison. Like bacteria, archaea also show the motility using archaellum and can be detected using hanging drop method. But due to obligate anaerobic nature of the organism, preparation should be done inside an anaerobic chamber. Study of morphological details at colonial and cellular level is an important criterion of taxonomic characterization of the methanogenic archaea and should be performed carefully. Use of transmission electron microscope (TEM) for the study of cellular morphology is a good option because along with cell morphology it also provides cytoplasmic details of the cells. Measurement of growth rate at different concentrations of NaCl, pH and temperature range and optima is a good indicator of physiological differences in closely related taxa and should be performed appropriately under similar laboratory conditions for better comparison. Optical density measurement as well as rate of methane generation can be used to monitor the growth of methanogens. In addition, solubility of hydrogen in the liquid medium is less in comparison with other gases; therefore, during the growth experiment of hydrogenotrophic methanogen, intermittent mixing of medium is required to supply the appropriate amount of hydrogen to the growing cells. Like bacteria and other members of archaea, data on mol% G + C, less than 70% DDH value and significant difference (at least 5%) in melting temperature of the hybrid DNA are recommended criteria for species delineation with substantial illustration of phenotypic characteristics of novel species of the methanogenic archaea.

Initially it was considered that the methanogenic archaea belong only to phylum Euryarchaeota, but discovery of the putative methane metabolic gene using the genome-centric metagenomics approach in Bathyarchaeota and Vettessatarckota indicated that the methanogens are widespread. According to the current survey of methanogenic archaea, there are more than 155 different species from 29 genera, 14 families, 8 different orders and four classes. Methanogenic archaea is mainly classified into two major classes, and these two classes are further separated by non-methanogenic lineages. Taxonomically, most of the characterized methanogens belong to phylum Euryarchaeota with few exceptions. According to current literature survey, phylum Euryarchaeota has seven orders, which include Methanobacteriales, Methanococcales, Methanomicrobiales, Methanosarcinales, Methanocellales, Methanopyrales and Methanomassiliicoccales (Whitman 2015). Despite all the efforts, major archaeal diversity has not yet been cultured and not available for study. Due to rapid growth in the culture-independent molecular metagenomic approach, now it is possible to retrieve the whole genome of the organisms from the environment without culturing them and verify their presence in any ecosystem using probes.

21.3 Role in Habitat and Environment

In spite of their obligate anaerobic nature, limited substrate range and slow growth rate, association of methanogens with a variety of ecosystems and environments has been reported. They are thus ubiquitously found on the terrestrial and aquatic environments. Several key features are common for all methanogens. However, there is a great diversity observed among methanogens. Anthropogenically methanogens exist within deep sediments, rumen of many herbivorous animals, intestines of many animals, insects and also humans. Significant diversity of methanogens has also been reported in extreme environments, viz. permanently cold Antarctic lakes, marine sediments associated without or with methane hydrates and hydrothermal vents. Methanogens are also present inside rocks, deep subsurface environments and environments characterized by extreme pH, salinity and nutrient limitation.

21.4 Methanogens of Animal Rumen and Intestinal Tracts

The rumen microbiota consists of a complex assemblage of anaerobic bacteria, anaerobic rumen fungi (ARF) and some protozoa that influence the health and productivity of animals. Methanogens within rumen are found to be diverse in nature. Rumen microbiota including methanogens is established within a short period after birth of the animal. Establishment of the methanogenic population initiates within 30 h in the rumen of lamb. The methanogenic population density reaches 10^9 organisms/g rumen fluid at ~ 1–3 weeks of age (Morvan et al. 1994; Skillman et al. 2004). Interestingly, colonization of methanogens is achieved even before the initiation of diet composed of forage material (Skillman et al. 2004). Methanogenic population in adult ruminants is usually dominated by the genus *Methanobrevibacter* (Janssen and Kirs 2008). Composition of methanogenic population in ruminant animals was reportedly unaffected by host types or geographic locations (Sundset et al. 2009a, b). It was reported that the levels of uncultured methanogens and *Methanobrevibacter ruminantium*-like methanogens were comparable in the rumen of sheep (Nicholson et al. 2007).

Order Methanobacteriales, Methanomicrobiales and Methanomassiliicoccales constitute the dominant methanogenic flora in the rumen of Murrah buffaloes in North India (Kumar et al. 2018). The study further revealed that *Methanobrevibacter* and *Methanomicrobium* were the dominant genera in three and one North Indian states respectively. The predominant methanogens in Murrah buffaloes (*Bubalus bubalis*) from India belong to the genus *Methanomicrobium* (Chaudhary and Sirohi 2009). *Methanobrevibacter* was the most dominant methanogen in the rumen of dairy cows (Kumar et al. 2015) and in bovine rumens (Miller and Lin 2002; Rea et al. 2007). Up to 5.5% of the bacterial population in the rumen of Norwegian reindeer was dominated by the genus *Methanobrevibacter* (Sundset et al. 2009a, b). The diversity of rumen methanogens is also well evidenced from recent cultivation reports of three genera of hydrogenotrophic methanogens, namely, *Methanobrevibacter, Methanobacterium* and *Methanocorpusculum*, from niches

like buffalo, blue bull, goat and sheep rumen, faeces and gut using BY medium (Joshi et al. 2018). Majority of studies also demonstrated the presence of methanogenic archaea in other ruminants. Different genera, viz. *Methanosarcina*, *Methanomicrobium* and *Methanobacterium*, are also commonly reported from bovine rumen (Jarvis et al. 2000). The *mcr*A gene sequence-based analysis revealed that Methanobacteriales, Methanosarcinales and Methanomicrobiales were the dominant orders of methanogens in ruminants (Sirohi et al. 2013).

21.5 Methanogens in Non-ruminant Animals

Just like in ruminants and humans, dominance of *Methanobrevibacter* can be observed in non-ruminants. Other *Methanobrevibacter* spp. were reportedly isolated from various non-ruminant animals. *Methanobrevibacter curvatus* RFM-2^T, *Methanobrevibacter cuticularis* RFM-1^T and *Methanobrevibacter filiformis* RFM-3^T have been isolated from termites' hindgut (Miller and Lin 2002). *Methanobrevibacter* was isolated from rat faeces (Maczulak et al. 1989). Shinzato et al. (1999) also reported more than 93% of archaea in the hindgut of the lower termites. In the termite *Reticulitermes speratus*, *Methanobrevibacter* species dominated. *Mbb. curvatus*, *Mbb. curticularis* and *Mbb. filiformis* were found to be associated with the hindgut of the termite *R. flavipes* (Guerrero 2001). Similarly, Sprenger et al. (2007) reported *Methanomicrococcus* from cockroach hindgut and *Methanosphaeracuniculi*1R7^T from rabbit rectum (Guerrero, 2001). Dominance of *Methanobrevibacter* in non-ruminants is also well evidenced from the recent cultivation reports on hydrogenotrophic methanogens from faeces of Indian star tortoise and termite gut (Joshi et al. 2018).

21.6 Methanogens in Humans

Methanogenic archaea are reported to be the members of human gut microbiota more than 30 years ago. Members of the order Methanobacteriales, *Methanobrevibacter smithii* and *Methanosphaera stadtmanae* are commonly reported from humans (Gaci et al. 2014). The association of methanogens with oral cavity and the GI tract has been reported in humans. Kulik et al. (2001) reported the genetic diversity of oral methanogens in human and stated that it is quite low and only three phylotypes exist. The major group of oral methanogens belong to *M. oralis* (Kulik et al. 2001), and *M. smithii* occur in the colon area (Miller et al. 1982). Methanogenic archaea are also a part of human skin microbiome and proposed to play a role in ammonia turnover. *Methanosphaera stadtmanae* MCB3^T and *Methanomassiliicoccus* are also reported from the human gut (Guerrero 2001; Dridi et al. 2012). Recently, Chaudhary et al. (2018) reported that the human microbiota harbours methanogenic species represented by *Methanobrevibacter smithii*, *Methanobrevibacter oralis*, *Methanosphaera stadtmanae*, *Methanomassiliicoccus luminyensis*, *Candidatus Methanomassiliicoccus intestinalis* and *Candidatus*

Methanomethylophilus alvus. Similarly, the archaeon *Methanobrevibacter smithii* strain KB11 was reported from a Korean faecal sample and was found to be a major colonizer of the human gut (Kim and Jeong 2018).

21.7 Methanogens in Marine Environments

Marine sediments harbour the largest deposits of methane on Earth, which produce 0.7–14 Tg methane year^{-1} (Valentine 2002, 2011). Generally, a major part of methane is produced biogenically by methanogens, and then methane oxidizers are known to further consume methane, the greenhouse gas. Hence, it is important to understand these organisms from all major subsurface sources with respect to methanogenesis. Sedimentary methanogens include representatives of the order Methanosarcinales, Methanomicrobiales, Methanococcales, Methanopyrales, Methanobacteriales and Methanomassiliicoccales (Katayama et al. 2016).

21.8 Methanogens in Sediments Associated with Permafrost

Methanogenic activity in Antarctic permafrost has been found to be dominated by *Methanosarcina* sp. which is well evidenced from reports from permafrost of the Bunger Hills Oasis (Vishnivetskaya et al. 2018). Similarly, *Methanosarcina mazei*, *Methanobacterium veterum* and *M. arcticum* are reported from Siberian permafrost (Rivkina et al. 2007; Krivushin et al. 2010; Shcherbakova et al. 2011). *Methanosarcina* has thrived and been preserved in the cold environment of Antarctic permafrost. The metabolic versatility allows *Methanosarcina* spp. to inhabit in a diverse array of environments including Siberian and Antarctic permafrost (Liu and Whitman 2008). Recently, Carr et al. (2018) have also reported that *Methanosaeta* an acetoclastic methanogen is found to be dominant at up to 14 mbs of marine sediments of Antarctica.

21.9 Methanogens in Sediments Associated with Methane Hydrates

Three methylotrophic methanogens, AK-4, AK-5 and AK-9, were isolated from methane hydrates of Skan Bay, Alaska. AK-4 was identified as *Methanosarcina baltica*. AK-5 and AK-9 possessed similar 16S rRNA gene sequence and were identified as close affiliates of *Methanococcoides burtonii*. The DNA-DNA hybridization studies revealed these two strains as novel species of genus *Methanococcoides* and were named as *Methanococcoides alaskense* sp. nov (Singh et al. 2005). Samples were collected from areas of occurrence of anoxic marine sediments at Hydrate Ridge, Oregon methane hydrates. Enrichments were set using formate, acetate or TMA as catabolic substrates. After 2 years of incubation at 4 °C to 15 °C, methane production was detected. Methanogens belonging to novel genera or

species within order Methanomicrobiales and Methanosarcinales were obtained. Analysis of the 16S rRNA gene libraries revealed no methanogenic sequence, indicating small proportion of methanogens in archaeal community (Kendall and Boone 2006). This was the first report of isolation of a methanogen from sediments associated with methane hydrate deposits in Nankai Trough off the eastern coast of Japan. The sediment core was collected at 247 m below the sediment. It was found to be closely related to *Methanoculleus marisnigri*. DNA-DNA hybridization experiments indicate a sequence similarity of only 49% due to which it was proposed as a new species, *Methanoculleus submarines* (Mikucki et al. 2003). Though in India there are three methane hydrates (Arabian Sea, western continental margin, Bay of Bengal), there are hardly any studies which focused specifically on the isolation of methanogenic archaea in methane hydrates. Therefore, only related studies are given. Dabir et al. (2014) recently reported draft genome sequence of a novel species of methanogenic genus *Methanoculleus*. This isolate was obtained from deep sub-seafloor sediment in the Krishna Godavari Basin off the eastern coast of India. This methanogen is thought to be mainly responsible for the production of methane that forms submarine methane hydrate deposits.

21.10 Methanogens in Hypersaline Habitats

A wide variety of hypersaline habitats are created because of desiccation of man-made as well as natural coastal environments. These habitats include transient salt pans, permanently hypersaline sabkhas, inland salt lakes and springs (Hovorka 1987). Gas and oil deposits as well as many industrial waste waters are commonly associated with hypersaline environment. Salt deposits containing brines from a cubic micrometer in volume to many cubic meters have also been found below the Earth's crust. Dissolution of ancient evaporates has resulted in formation of large lakes on the floor of the Gulf of Mexico, Mediterranean Sea and Red Sea. Methane is the major gas detected in the Martian atmosphere by the NOMAD instrument (Vandaele et al. 2015). Recent studies have shown that the sabkhas and Martian are similar (McKay et al. 2016). Hence, studies on the effects of extreme conditions including hypersaline environment on methanogenesis have generated a lot of interest.

Salinity is one of the major components that affect microbial community. In high sulphate-containing environments such as saline and hypersaline habitats, sulphate-reducing bacteria outcompete methanogens as they have higher affinity for growth substrates such as hydrogen, formate and acetate (Lovley et al. 1982). However, microbial metabolism leading to the production of methane is still an important process in salt-rich environments in deep zones characterized by sulphate depletion (Wilms et al. 2007), with excess hydrogen production (Hoehler et al. 2001; Buckley et al. 2008) and where non-competitive carbon sources are available (Oremland et al. 1982; Winfrey and Ward 1983). Such carbon sources that cannot be used by sulphate-reducing bacteria include methanol, methylated amines and methylated sulphides. As a result methylotrophic methanogenesis is almost always the

dominant methanogenic process in saline and hypersaline environment (Oremland and King 1989). Methylated sulphides as well as amines are commonly derived from salinity-induced compatible solutes such as dimethylsulphoniopropionate and glycine betaine and hence are present in surplus concentrations in marine and hypersaline environments (Kiene et al. 1986; Kiene and Visscher 1987; Curson et al. 2011). Recent studies have shown that few marine strains of the genus *Methanococcoides* are capable of utilizing choline and glycine betaine as substrates for methanogenesis without the help of a syntrophic partner (L'Haridon et al. 2014; Watkins et al. 2014). One *Methanococcoides* strain studied in detail was shown to only partially demethylate glycine betaine to N,N-dimethylglycine, possibly because the product may also serve as a compatible solute and also because much more energy is gained from the first demethylation step than subsequent steps (Watkins et al. 2014). In hypersaline environments, the direct use of glycine betaine as a substrate for methanogenesis has not been demonstrated yet. Studies on methanogens isolated from saline environments show that methylotrophic methanogens contribute to about 30%, hydrogenotrophic methanogens contribute approximately 12%, while only 4% were acetoclastic methanogens highlighting contribution of methylotrophic substrates to methanogenesis at different salinities (Oren 2011). This can also be explained from the fact that the net energy gained per mole of methylotrophic substrates is higher as compared to hydrogenotrophic and acetoclastic substrates. In order to maintain osmotic balance, halophiles need to spend additional energy which can be fulfilled via methylotrophic methanogenesis (Oren 2011). Recently, a fourth (hybrid) pathway of methanogenesis has been proposed (Borrel et al. 2014). Sorokin et al. (2017) observed that when sediment slurries from hypersaline soda lakes were incubated under high pH and temperature as well as salt-saturating conditions in the presence of methanol or trimethylamine + formate or H_2, methanogens followed a novel methyl reduction pathway. It involves methylation of single carbon (C1) compounds which serve as electron acceptors, while H_2 serves as an electron donor. Based on its ribosomal protein phylogeny, the novel methanogens are most closely related to the class Halobacteria and are now classified to form a novel class 'Methanonatronarchaea' (Sorokin et al. 2017). It has also been suggested that this pathway may outcompete both the classical methylo- and hydrogenotrophic methanogenic pathways in salt-saturated conditions (McGenity and Sorokin 2018).

21.11 Methanogens from Petroleum Reservoirs

The history of the oil business goes as far as 1859 when Edwin L. Drake invented a way to drill an oil well in Titusville, Pennsylvania. Crude oil has played an important role in the development and progress of human culture. On account of low availability of nutrients, high pressure, temperature and salinities, deep subsurfaces like petroleum reservoirs were considered to be too hostile for survival of microorganisms and hence were thought to be sterile. Pioneering studies by Bastin et al. (1926) and Claude ZoBell (Bass 1999) changed this perspective. Application of

culture-independent methods further augmented our outlook regarding the microbial biodiversity. On account of low redox potential, obligate anaerobic and facultative bacteria and archaea harbour oil reservoirs. Based on availability of electron donors and acceptors, sulphate reducing bacteria, methanogens, fermentative microbes, syntrophs, nitrate, manganese and iron reducers have commonly been reported (Varjani and Gnansounou 2017). Geological evidence suggested that the major portion of the Earth's petroleum reservoirs has been degraded by microorganisms over the millennia, leading to the formation of methane (Hallmann et al. 2008; Jones et al. 2008). A number of reports on hydrocarbon metabolism under methanogenic environments have been published under laboratory conditions (Gieg et al. 2010; Toth and Gieg 2018). Hydrogenotrophic, acetoclastic as well as methylotrophic methanogens have been reported from oil reservoirs worldwide. Except for the order Methanopyrales, methanogens belonging to the rest all the four orders have been described so far. As a result of technical and practical difficulties to sampling, most of the culture-dependent and culture-independent diversity studies are carried out using either formation water or produced water obtained from well heads of oil wells. Members from the order of Methanosarcinales, Methanobacteriales, Methanococcales and Methanomicrobiales contribute a major fraction of oil reservoirs of archaeal communities. Apart from these members of the order Methanocellales, Methanomassiliicoccales have also been reported.

21.12 Hydrogenotrophic Methanogens

Many novels as well as previously described strains of hydrogenotrophic methanogens growing at temperatures in the range of 20–80 °C have been reported in oil wells (Birkeland 2004; Kim et al. 2018). Novel mesophilic hydrogenotrophic methanogens such as *Methanoplanus petrolearius* (Ollivier et al. 1997) and *Methanoculleus receptaculi* (Cheng et al. 2008) have been reported from African and Chinese oil fields, respectively. A mesophilic halotolerant hydrogenotrophic methanogen, *Methanocalculus halotolerans*, has also been recovered from a French reservoir (Ollivier et al. 1998). Besides these, members of the genera *Methanobacterium* and *Methanoculleus* have also been reported in enrichment set-up using low salinity oil reservoir fluids (Orphan et al. 2000). A novel methanogen, *Methanobacterium ivanovii*, has also been isolated from oil wells in Tatarstan (Belyaev et al. 1986). Members of the genus *Methanothermobacter* have frequently been reported from many high-temperature oil wells (Jeanthon et al. 2005). Recently two novel thermophilic and hydrogenotrophic methanogens belonging to the genus *Methanothermobacter*, viz. *M. crinale* and *M. tenebrarum*, had been isolated from Chinese and Japanese oil wells, respectively (Cheng et al. 2011; Nakamura et al. 2013). Other hydrogenotrophic members of the genera *Methanoculleus* (Orphan et al. 2000; Cheng et al. 2008), *Methanococcus* and *Methanothermococcus* (Nilsen and Torsvik 1996) have also been described from thermophilic oil wells.

21.13 Methylotrophic Methanogens

Methylotrophic methanogens have mostly been isolated from oil wells having temperatures below 60 °C. Novel mesophilic methylotrophic methanogens such as *Methanosarcina siciliae* (earlier *Methanolobus siciliae*) from gas and oil well from Gulf of Mexico (Ni and Boone 1991) and *Methanosarcina mazei* LYC (Liu et al. 1985) from sediment from an oil exploration drilling site in Israel have been isolated. In addition, a halophilic methanogen, *Methanohalophilus euhalobius* (Obraztsova et al. 1987a, b), had also been reported from the Western Siberian oil reservoir. A thermophilic methylotrophic isolate, namely, *Methermicoccus shengliensis*, representing a novel family of Methermicoccaceae had been reported (Cheng et al. 2007). Members of the order Methanomassiliicoccales have also been reported from oil reservoirs (Vigneron et al. 2017).

21.14 Acetoclastic Methanogens

Active mesophilic (Grabowski et al. 2005) as well as thermophilic (Bonch-Osmolovskaya et al. 2003) acetoclastic enrichments have been established from oil reservoirs. But till date only a single isolate, *Methanosarcina mazei*, had been reported from oil fields in Tatarstan, Russia (Obraztsova et al. 1987a, b).

In general, hydrogenotrophic methanogens isolated from oil reservoirs outnumber acetoclastic methanogens. The underlying reason for this could be attributed to the standard changes in free energies for methane production. Thermodynamically hydrogenotrophic methanogenesis is a more favourable reaction as compared to acetoclastic methanogenesis as can be seen from Eqs. 21.1 and 21.2.

$$4H_2 + CO_2 \rightarrow CH_4 + 2H_2O \; \Delta G^{o'} = -135.1° \, kJ° \, mol° \, CH_4^{-1} \tag{21.1}$$

$$CH_3COOH \rightarrow CH_4 + CO_2 \; \Delta G^{o'} = -36.0° \, kJ° \, mol° \, CH_4^{-1} \tag{21.2}$$

Hence, the evolutionary forces have naturally selected hydrogenotrophic methanogens in abundance as compared to acetoclastic methanogens (Garcia et al. 2000).

21.15 Environmental Impact of Methanogens

Methanogens are considered to be the most primitive microorganisms on the Earth. They are helpful as well as harmful to the environment. Their role in the anaerobic decomposition is in the terminal stage to convert C-1 compounds into methane and carbon dioxide. In doing so, methanogens return carbon particularly from anoxic environments to the atmosphere and thus play a vital role in the carbon cycle. At the same time, the emission of methane from land and marine environment contributes to global warming and climate change.

Methanogens are the largest producers of methane. More than 60% of the global methane is produced by methanogens as the end product of their anaerobic metabolism. Methane is considered as a potent greenhouse gas. Source of methane in the atmosphere is either natural or anthropogenic. Methane produced and emitted in wetland, marines, rivers, lakes, etc. is considered as natural methane. Under anthropogenic methane, main sources are fossil fuels, ruminants, rice fields, anaerobic treatment plants/digesters, landfills and several others. Except methane in oil and gas strata and of methane hydrate, most of methane is real-time methane produced by methanogens during anaerobic decomposition of organic matter. Methanogens also become an extremely important group of microorganisms valued by the industry because of their applications in the production of bioenergy. Methanogens have now been considered extremely important in the treatment of high-strength industrial waste as the energy produced by the methanogens in the form of methane gas has made the waste treatment a cost-efficient process rather than a cost-intensive process. Methane is a major inflammable gas in natural gas. It is well accepted as a gaseous fuel at domestic and industrial level. Similarly methane produced during anaerobic digestion of organic matter in biogas plants and anaerobic treatments of wastewater is nowadays commonly used for electricity generation or as a gaseous fuel. Methane, if not properly captured and used, gets emitted into the atmosphere. Such methane is a major trace gas in the Earth's atmosphere and responsible for global warming. Methane hydrate deposits are considered to be responsible for 4–5 Tg of yearly methane emission to the atmosphere. Methane produced both in fermentation and landfills often reach the stratosphere. Here it reacts with free radicals to form CH_3^+, which can then result in both production and destruction of ozone. Further, methane absorbs infrared radiation which can thus contribute to global warming. On global scale, anthropogenic sources contribute more methane than the natural sources. Total global methane emissions amount to approximately 582 Tg methane per year. Natural sources contribute approximately ~200 Tg (Denman et al. 2007) with rest contributed by the anthropogenic sources. Interestingly 581 Tg of these methane emissions is consumed in global methane sinks every year. Thus, the current increase in atmospheric methane concentrations amounts to only 1 Tg/year. However, possibility of enhanced natural methane emissions in the future due to climate change poses a serious threat of increased global warming and may negate attempts at mitigation of greenhouse gas emissions. Methanogens have another important role to play in the recycling of the global carbon. They recycle carbon through anaerobic decomposition of organic matter to the aerobic environments.

Methane is the second most important anthropogenic greenhouse gas after CO_2. It is a strong greenhouse gas. When compared at molecule level, methane is 20 times as potent as greenhouse gas as carbon dioxide. Both these gases are responsible for global warming which has changed the climate on the Earth. The effects of global warming have started now. In years to come, these will worsen global climate, global water and food scenario and world economics and politics, too.

Methane emission from all these sources is ever increasing and hence is the concern. However, atmospheric methane data reported that when compared with estimated terrestrial and marine methane data, it becomes clear that much more data collection is required. Moreover, the methane emission data at high resolution with reference to time and space is essential. Use of isotope in methane measurement shall be very useful. With increasing awareness about environmental pollution and shortage of fossil fuel as well as increasing paddy cultivation and cattle population to meet demands of fuel and food of increasing global population, generation of anthropogenic methane will increase in years to come.

Methanotrophs do consume methane produced in soil, rice fields and cattle waste-based biogas plants. Similarly, anaerobic methane oxidation has also been reported. However, the activity of these bacteria is not enough to consume methane form air in substantial quantity. At present, the rate of methane oxidation is too low compared to the rate of methane emission from aquatic and terrestrial environments. Therefore, much research is required on microbial methane oxidation on a large scale. The same is true for anaerobic methane oxidation. Microbiologists need to think and act to curb methane production in intestinal systems including ruminants if methanogens have no other role for host health.

21.16 Methanogens as Boon

The flammable gas methane produced by methanogens is considered to be a sustainable source of an alternate energy for the future (Ren et al. 2008). Methane can be considered suitable:

1. As vehicular fuel.
2. For power generation.
3. For synthesis of valuable chemicals.
4. As CNG (Ren et al. 2008).
5. As a substrate for methanotrophs which can metabolize methane into SCP, enzymes, PHB or methanol (Ge et al. 2014; Strong et al. 2015).

The biological production of methane from industrial, agricultural or domestic waste is considered as a techno-commercially feasible process for the sustainable and renewable energy generation. Interestingly, production of methane using electrochemical processes is more energy-efficient [< 0.3 kWh/$m^3$of methane (0.16 MPa, (Bär et al. 2015)] when compared with biological methane generation. The biomethanation process is, however, desired because of its higher tolerance against impurities (H_2S and NH_3) (Bär et al. 2015). Extensive research is going on to improve the efficiency and yield of the biomethanation process. Biomethanation is commonly used in sewage and wastewater treatments. Biomethanation process being anaerobic has certain advantages over aerobic treatment processes. Therefore, when wastewater is large in quantity and high strength with respect to organic load, biomethanation becomes a little costlier. Moreover, it produces fuel gas in the form

of methane. Methanogens are also being explored for unconventional applications such as electromethanogenesis (Blasco-Gómez et al. 2017). Methanogens are a diverse group of organisms that could grow over a wide range of ecophysiological conditions such as extremely high or low temperatures/high osmolarities/pH values. Hence, it is important to optimize industrial biomethanation processes.

Applications of methanogens are briefly described below.

21.17 Hydrogen Production

Several methanogens have been reported to produce hydrogen (Valentine et al. 2000; Goyal et al. 2016). Methanogens, under hydrogen-starved environment, divert metabolism towards production of hydrogen instead of hydrogenotrophic metabolism. Formate and other metabolites but not methane have been found out to be the source of hydrogen (Valentine et al. 2000; Lupa et al. 2008). *Methanothermobacter marburgensis*, *Methanosaeta thermophila* and *M. barkeri* produced ~ 0.25, 0.23 and 0.21 μmol hydrogen/mg cell dry mass, respectively (Valentine et al. 2000). Application of methanogens to produce hydrogen has not yet been commercialized and is mostly restricted to lab-scale studies. Hydrogen production in methanogens is presumably catalysed by the hydrogenases enzymes present in methanogens (Valentine et al. 2000).

21.18 Methane from Oil and Coal Beds

Conventional methods of oil recovery from oil reservoirs can extract a maximum of up to 30 to 40% of the oil in place with nearly two-third remaining un-extracted (Gieg et al. 2008). One of the approaches of extracting residual energy from such depleted reservoirs is to convert residual oil into methane with the help of consortium of microorganisms consisting of oil-degrading bacteria and methanogens. Gieg et al. (2008) developed a microbial consortium consisting of members of *Clostridiales*, *Bacteroidetes* and *Chloroflexi*, syntrophic sulphate-reducing bacteria and methanogens such as *Methanosaeta* sp. This consortium was developed from subsurface sediments. Methane yield obtained using this consortium was 3.14 mmol/g crude oil in studies done in sand pack column. Biomethanation of oil has been attempted from oil fields, oil sands tailing ponds and others (Voordouw 2011). Coal bed methane has also been exploited as a source of natural methane. Less than half of this methane is of biogenic origin. Members of the Order Methanosarcinales have been reported to be the dominant methanogens contributing to the formation of coal bed methane. Interestingly, methoxylated aromatic compounds serve as a substrate in this environment (Sakata et al. 2017). *Methermicoccus shengliensis* was reported to produce up to 10.8 μM methane per g coal in this study. Coal bed methane, unlike biomethanation of crude oil, is a commercially viable process and is being practiced in the field.

21.19 Biomethanation of Organic Substrates

Biomethanation of organic substrates such as proteins, lipids and polysaccharides consists of the following four steps: (1) Hydrolysis – in this step, the complex organic matter is hydrolysed by the enzymes such as protease, lipase, cellulase, amylase, etc. into monomers or oligomers. Such metabolites including amino acids, fatty acids, sugars, etc. are used by the acidogens as substrates or carbon and energy source. (2) Acidogenesis – in this step, oxidative microbial metabolism leads to formation of organic acids such as butyrate, propionate, acetate, formate, ethanol as well as gasses like H_2 and CO_2. (3) Acetogenesis – in this step, microbial metabolism leads to formation of end products which mainly include acetate and CO_2. (4) Methanogenesis – in this step, methanogenic metabolism converts acetate (and methylated compounds) as well as CO_2 and H_2 to methane. It is important that all four steps, i.e. hydrolysis, acidogenesis, acetogenesis and methanogenesis, operate at a balanced rate for the stable biogas production. Operation of all four steps at a balanced rate could be ensured by optimizing the operational parameters such as temperature (Vanegas and Bartlett 2013), hydraulic retention time (Rincón et al. 2008), pH (Lay et al. 1997) and nitrogen source (Karakashev et al. 2005). Biomethanation plants operated on commercial scale in Europe include municipal solid waste, sludge, agricultural waste/residue and others. Biogas processes are expected to produce up to 25% of the bioenergy used (Holm-Nielsen et al. 2009). Over the years, environmental engineers have evolved different designs of biomethanation plants or anaerobic treatment plants as they are commonly called. The earlier designs were simpler. With better understanding of microbiology of biomethanation process and particularly physiology and biochemistry of methanogens, advanced designs of anaerobic treatment plants are developed. These pay more attention to avoid wash out methanogens as they are slow grower than non-methanogens, for example, anaerobic filters. In yet another design, namely, upflow anaerobic sludge blanket (UASB), gas formation in the granular methanogenic sludge is used to keep all the essential bacteria and methanogenic archaea with each other and also come in good contact with the wastewater being treated.

21.20 Biomethanation of Sewage

Biomethanation of sewage leads to generation of renewable energy as well as clean water. Organic matter in the sewage is degraded through the hydrolysis, acidogenesis and acetogenesis phases. The last step is contributed by the methanogens, metabolism of which results in the extraction of energy in the form of methane. The process has several advantages as it reduces the volume of sludge to be disposed as well as pathogen load. Furthermore, biogas generated can be used as a fuel or for electricity generation. Another advantage of the process is the prevention of greenhouse gas (methane) emission to environment. Uncontrolled degradation of sewage may result in release of greenhouse gases into the environment (Enzmann et al. 2018).

21.21 Biomethanation of Agricultural Waste

The agricultural waste includes agro-residue such as straw silage, animal manure and slurry generated in farms cultivating pigs, poultry, fish, cattle, etc. Large part of the agricultural waste is biodegradable. Biomethanation of such high-volume biodegradable waste makes the process energy-efficient as well as a profit-making venture rather than a cost-intensive waste treatment. Further, biomethanation of the agro-waste also reduces odours as well as pathogen load. The slurry generated during the biomethanation process has potential application as an organic fertilizer. Agriculture sector can also offer other substrates for energy recovery via biomethanation route. For example, sugar beet or maize silage can serve as renewable substrates for sustainable biogas production (Demirel and Scherer 2008a, b; Lebuhn et al. 2008). It is important to note that the lignocellulosic plant material in many cases needs thermochemical pre-treatment which can increase the process cost and reduce the biomethanation potential. However, some of the recent studies have shown that use of anaerobic fungi may eliminate the need for such pre-treatment because of their ability to produce cellulosomes, a complex of lignocellulolytic enzymes that enables microbial attack and hydrolysis of lignocellulose complex. This ensures efficient biodegradation of the organic component and efficient generation of the metabolite in cattle waste biogas plant.

21.22 Future Perspectives

Despite the tremendous potential, biomethanation process has not been exploited on commercial scale to its fullest potential. This can be attributed to lack of adequate understanding of the methanogenic microbial metabolism of the complex organic substrate. However, with the recent advances in the sequencing technologies, it has become increasingly possible for us to gain insights into microbial metabolism, understand the composition of the microbial population residing within biogas digesters, identify novel and more efficient microbes, etc. Recent technological advances have also provided us more efficient diagnostic tools to monitor the reactor performance. It is the combination of above that has the potential to improve and increase the reach of the biomethanation processes for industrial application or for commercialization even in the domestic sector. Some of the most important achievements have been to avoid overloading of the digester or underperformance of the anaerobic biomethanation processes. Process optimization and automation in process monitoring are expected to increase the number and energy generation capacity of the biomethanation plants. This will not only increase the application of biomethanation process at industrial scale but will also improve the acceptability of biogas plants at domestic scale. Advanced biomethanation plants are expected to generate biogas that can be used as a vehicular fuel (in the form of bioCNG), electricity generation and also for the production of valuable chemicals. In addition, the biogas plants will play an important role in nutrient recycling in the form of organic fertilizers generated as sludge during the biomethanation process.

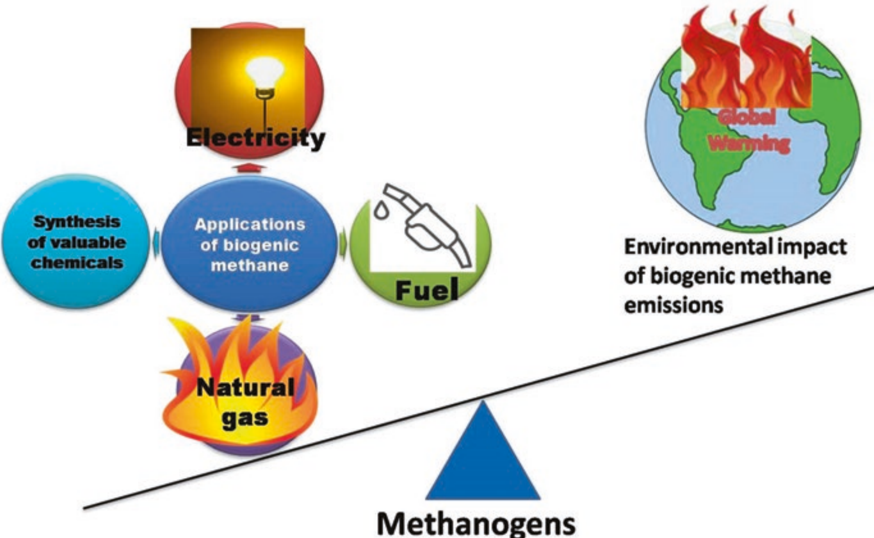

Fig. 21.3 Applications of biogenic methane outweigh the adverse impact of methane emissions

Besides heat and electricity, methane will be extensively used for the production of speciality chemicals using specific microorganisms (e.g. methanotrophic bacteria). Some of the valuable products that could be synthesized using methanotrophic microbes and methane as substrate include SCP, EPS, biological/biodegradable plastics and organic acids such as succinic acid or lactic acids (Kougias and Angelidaki 2018). However, it is important to note that these processes are being explored at laboratory scale at present, and there is a need to scale up the processes.

21.23 Conclusions

The foregoing discussion underscores that the adverse environmental impact of the methanogenic potential is significantly outweighed by the application of methanogens in industry for the generation of heat, electricity, fuel as well as industrially valuable chemicals (Fig. 21.3). Thus, it can be safely said that methanogens in today's world and in the future are a boon rather than a bane.

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Genetic Diversity of Pathogenic Yeasts

22

Kunal Ranjan and Marcio José Poças-Fonseca

Abstract

Human fungal diseases, including yeast infections, pose a significant concern to public health, affecting over one billion people worldwide and causing one million deaths per annum. Yeasts do not form a separate taxonomic group; instead, they are placed in two separate phyla: *Ascomycota* and *Basidiomycota*. Yeasts occur in a variety of environmental niches; many are part of the human normal microbiota but become pathogenic in immunocompromised individuals, thus acting as opportunistic pathogens. Since the number of people presenting immune system dysfunctions is increasing, yeast pathogens are becoming much more prevalent in systemic infections. Pathogenic yeasts include species of *Cryptococcus*, *Candida*, *Paracoccidioides*, *Histoplasma*, *Sporothrix*, *Blastomyces* and *Pneumocystis*. These yeasts cause a significant challenge for clinical microbiologists and physicians. Advances in medical research, made during the last few decades, have improved the diagnostic and therapeutic capabilities for a variety of infections. The availability of refined molecular data has increased the understanding of genetic and environmental diversity of pathogenic yeasts, as well as of the evolution of virulence attributes. This chapter reviews the genetic diversity and geographical distribution of the aforementioned pathogenic yeasts as assessed by specific loci targeting techniques.

Keywords

Opportunistic yeast pathogens · Genetic diversity · *Cryptococcus* · *Candida* · *Paracoccidioides* · *Histoplasma capsulatum* · *Sporothrix schenckii* · *Blastomyces dermatitidis* · *Pneumocystis jirovecii* · *Penicillium marneffei*

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22.1 Introduction

Yeasts are fungi that grow as single cells which are generally characterized by the presence of coenocytic hyphae. They reproduce either by budding or by binary fission. The occurrence of a sexual reproduction cycle was described for some species. Yeasts do not form a separate taxonomic group; instead, they are placed in two separate phyla: *Ascomycota* and *Basidiomycota*. Some fungi have the ability to switch from the yeast to the hyphal phase under certain environmental conditions, like temperature, humidity, CO_2 tension and pH; these are called dimorphic fungi. Since yeasts are chemo-organotrophs, they use organic compounds as energy source and do not require sunlight for growth. Yeasts occur in diverse environments.

Fungal infections are gaining undue attention, largely due to their increasing incidence, mainly in organ transplanted, cancer chemotherapy, diabetic and HIV/AIDS patients. Despite the countless yeast cells and fungal spores in the environment, only a few (about 200 species) directly affect human health (Richardson and Warnock 2012). This is due to the fact that most of the fungi cannot grow at the mammalian body temperature. Nonetheless, yeast infections, particularly the systemic ones, represent a significant challenge for researchers and physicians. It is estimated that fungal infections, including yeasts, cause over a million deaths per annum; these are due to mainly species of *Cryptococcus*, *Candida* and *Aspergillus* which affect immunocompromised individuals (Brown et al. 2012). Many fungi can grow under environmental stress by forming sclerotia, a dormant resting body that helps the spores to search for new favourable habitats over long distances (Wang and Lin 2012), while others can switch from yeast to hyphal growth in order to invade animal tissues (Nemecek et al. 2006).

Generally yeasts are part of the normal microbiota as commensal organisms. However, they can become pathogenic in different body parts and organs in the immunocompromised individuals. This chapter focuses on the human pathogenic yeasts *Cryptococcus*, *Candida*, *Paracoccidioides*, *Histoplasma capsulatum*, *Sporothrix schenckii*, *Blastomyces dermatitidis*, *Pneumocystis jirovecii* and *Penicillium marneffei*. Genetic diversity, epidemiology and geographical distribution are addressed by specific loci targeting techniques.

22.1.1 Cryptococcus

Cryptococcus is a basidiomycete genus and normally lives as single cells, although some species may form biofilms. It was isolated for the first time by Francesco Sanfelice from fermented peach juice samples in 1894 (Sanfelice 1894). Since then, it was described worldwide, from the tropics to the Arctic and Antarctic regions. Different species are able to colonize plants (Jager et al. 2001; Li et al. 1995), different kinds of feces, particularly from birds (Mancianti et al. 2002), lakes (Rosa et al. 1995) and marine environments (Kutty and Philip 2008). This genus has been described as a dominant fungal group in soils (Valinsky et al. 2002). Thirty-seven species of *Cryptococcus* have been described; most of them are not pathogenic. *C.*

neoformans and *C. gattii* are the major pathogens to humans and animals, while *C. alurantii* and *C. albidus* can rarely cause diseases.

Cryptococcus neoformans and *C. gattii* were subtyped by PCR fingerprinting, AFLP and MLST. In PCR fingerprinting, amplification produces a banding pattern that discriminates at the subspecies level. According to this typing method, *C. neoformans* was divided into four variants. Out of the four variants, three (VNI, VNII and VNB) represent serotype A, which comprises *C. neoformans* var. *grubii*. On the other hand, VNIV corresponds to *C. neoformans* var. *neoformans* (serotype D). *C. gattii* was also grouped into four variants: VGI and VGII (serotype B) correspond to *C. gattii* var. *gattii* and *C. gattii* var. *deuterogattii*, respectively; VGIII and VGIV belong to serotype C (*C. gattii* var. *bacillisporus* and *C. gattii* var. *tetragattii*, respectively) (Chen et al. 2014).

The AFLP typing method involves the digestion of DNA with mainly the endonucleases *Mse*I and *Eco*RI, followed by PCR amplification that generates fragments from many different genomic sites, which are then resolved by polyacrylamide gel electrophoresis. With this method, six AFLP genotypes were identified: AFLP1, AFLP2 and AFLP3 correspond to *C. neoformans* while AFLP4, AFLP5 and AFLP6 to *C. gattii*. The AFLP1 and AFLP6 genotypes were further subdivided into AFLP1A/AFLP6A and AFLP1B/AFLP6B. AFLP6A was described as more virulent than AFLP6B (Boekhout et al. 2001; Cogliati 2013).

The MLST (multilocus sequence typing) genotyping technique is based on the sequence analysis of different allele variants of selected loci. Seven *Cryptococcus* loci, some of them involved in the virulence attributes, are normally included in standard MLST genotyping analyses: *GDP1*, *IGS1*, *URA5*, *CAP59*, *LAC1*, *SOD1* and *PLB1*. MLST typing of 230 *C. neoformans* isolates from South African HIV-positive patients revealed great genetic diversity illustrated by 50 distinct sequence types (STs), including 18 novel STs. Forty-six distinct STs were found within patients living in Cape Town only. Mating types were also identified from 229 isolates. Two hundred twenty-six isolates were identified as *MATα* and the remaining three as *MATa* (Beale et al. 2015).

Cryptococcus complexes representing hybrids between *C. neoformans* and *C. gattii* have also been reported, such as VGI–VNI and VGII–VNIV (Byrnes et al. 2011; Farrer et al. 2016; Ding et al. 2016). These hybrids contribute to the genetic variability reported to date for the *Cryptococcus* genus.

An accurate estimation of a cryptococcal infection burden is essential for prevention and controlling strategies. Infection of the central nervous system by *Cryptococcus* yeasts is the third most frequent neurological complication in AIDS patients. Cryptococcal meningitis results in 15% of AIDS-related mortality (Cogliati 2013). Park et al. (2009) estimated the incidence and death rates due to cryptococcal infection from published studies of the pre-ART (antiretroviral therapy) era. It was estimated that each year 957,900 cases of cryptococcal infection occurred among people infected with HIV; about 624,725 of these patients died. Sub-Saharan Africa was the most affected region of the globe, with approximately 504,000 deaths. From UNAIDS 2014 data (http://www.unaids.org/en), it was estimated that 31.8 million adults lived with HIV globally, 21.7 million of them in sub-Saharan Africa.

Rajasingham et al. (2017) have recently reviewed the annual *Cryptococcus* infection numbers in the world for the year 2013. Cryptococcal antigenaemia was prevalent in 278,000 people, and among them 223,100 developed cryptococcal meningitis, 73% of them in sub-Saharan Africa. Asia and Pacific comprised 19% of the global meningitis incidence.

Globally around 180,000 deaths occur annually due to cryptococcal infection; about 136,000 cases in sub-Saharan Africa (Rajasingham et al. 2017). *C. neoformans* accounts for 80% and *C. gattii* for 20% of the total cryptococcosis incidence. *C. neoformans* molecular type VNI is the most dominant causative agent (63%), followed by VNII/VNII (6% each) and VNIV (5%). Considering *C. gattii* infections, molecular-type VGI is the most common (9%) followed by VGII (7%), VGIII (3%) and VGIV (1%) (Meyer et al. 2011).

About 20,000 *C. neoformans* and *C. gattii* isolates were reported from different African countries. These were isolated from different bird excreta, soil samples, house dust, trees and even from South African cheetah affected by cryptococcosis (Millward and Williams 2005; Litvintseva et al. 2011; Mseddi et al. 2011). From these African isolates, 68% corresponds to VNI, 13% to VNB, 11% to VNII and 1% to VNIII. Interestingly, the VNIV type was not found in any of the isolates (Litvintseva et al. 2005; Cogliati 2013). Out of 1439 isolates from Brazil, the majority belonged to VNI, followed by VGII, while the VGIV type was not detected (Cogliati 2013).

C. neoformans/*C. gattii* infection ratio varies according to the continent: 68:1 in Europe, 33:1 in Africa, 7.6:1 in Asia, 4.5:1 in Central and South America, 3.5:1 in North America and 1:1.5 in Oceania. Maybe due to the difficulty in identifying the *Cryptococcus* complex (hybrids between *C. neoformans* and *C. gattii*), only three cases were reported in the Netherlands, one in India, Brazil, Colombia and Canada (Cogliati 2013). Singer et al. (2014) isolated cryptococcal strains from cats and dogs with cryptococcosis: VGIII was more prevalent in cats while VNI in dogs. Phylogenetic studies also revealed that these isolates are very closely related to strains isolated from humans.

The increasing occurrence of cryptococcal infections in immunocompetent individuals seems to be related not only to genetic recombination, as illustrated by the disseminated outbreak of cryptococcosis in the southwest cost of Canada and Pacific northwest cost of the USA (Harris et al. 2012), but also to epigenetic-driven phenotype plasticity (Brandão et al. 2015, 2018).

22.1.2 Candida

The *Candida* genus belongs to *Ascomycota* that represents a common cause of fungal infections worldwide. Over 17 species cause infection. Nonetheless, 90% of the infections are caused by *C. albicans*, *C. glabrata*, *C. parapsilosis*, *C. tropicalis* and *C. krusei*. *Candida* species are present in healthy human mucous oral cavity and gastrointestinal and urogenital tracts as commensal organisms of the normal microbiota. In immunocompromised individuals, they can cause a variety of clinical

manifestations, from mucocutaneous overgrowth to bloodstream infection. Three types of candidiasis are normally described: (1) infection of the mouth, throat and esophagus, (2) vaginal candidiasis and (3) invasive candidiasis (Eggimann et al. 2003).

Candida albicans is the major cause of fungal infection in humans; it can transit between yeast, pseudo hyphae and true hyphae under different signals, such as the presence of serum. It is diploid and unable to undergo typical meiosis, although mating leads to the production of tetraploids. For heterothallic mating (between *a* and α cells), cells must undergo an epigenetic phenotypic switch from the white (sterile) to the opaque (mating competent) state, with the help of pheromone signalling pathways. Homothallic mating (a-a or α-α) has also been reported in laboratory conditions, after the deletion of the *BAR1* gene, and it also generates tetraploids. The tetraploid cells then undergo chromosomal rearrangements, reassortment of the genome via chromosomal gain and loss, gene deletions and translocations which result in significant genetic diversity that may be advantageous under stressful conditions or fluctuating environment (Alby et al. 2009; d'Enfert et al. 2017).

More than 50% of all the candidaemia cases in Europe are caused by *C. albicans*, followed by infections by *C. glabrata* and *C. parapsilosis* (14% each), 7% by *C. tropicalis* and 2% by *C. krusei* (Tortorano et al. 2006). In Brazil, 40.9% of the cases of candidaemia are caused by *C. albicans*, followed by *C. tropicalis* (20.9%), *C. parapsilosis* (20.5%) and *C. glabrata* (4.9%). Changes in epidemiology were observed in other Latin American countries like Colombia, Ecuador and Venezuela (Nucci et al. 2010). Among the 100 diabetic patients registered in the diabetics clinic of the Safdarjung Hospital, New Delhi (India), *C. albicans* was responsible for 51% of the infection cases; *C. tropicalis* and *C. krusei* accounted for 15% and 14%, respectively; and the remaining were due to other non-*C. albicans* infections (Kumar et al. 2014).

The genetic diversity of *C. albicans* had been assessed mainly by three methods. DNA fingerprinting by Southern blotting with the probe Ca3, which contains repeated sequences, read throughout genome that allows the discrimination and quantification of genetic distances between unrelated strains. The Ca3 fingerprinting method enabled the clear distinction of *C. albicans* in five clades: groups I, II, III, SA and E (Pujol et al. 2002).

Cluster analysis of 1410 *C. albicans* isolates by MLST revealed five main clades based on the geographical location, namely, clades 1, 2, 3, 4 and 11 and the additional clade 13. North American isolates were clustered in clades 1 and 3, while all the South American isolates were assigned to clade 8. Clade 1 isolates were described as the most effective human colonizers. African isolates were clustered to clade 4. Clade 13 was distantly related to all the other five clades and corresponded to isolates from Eastern Asia (Odds 2009; Shin et al. 2011).

MLST microsatellite typing shows high discriminatory power when a well-defined combination of microsatellites is used. d'Enfert et al. (2017) employed 11 microsatellite markers (*CDC3*, *HIS3*, *CAI*, *LOC4*, *ZNF1*, *CAIII*, *KRE6*, *CAV*, *CAVII*, *CAREBEME* and *CEF3*) to evaluate *C. albicans* genome diversity and dynamics. *HIS3* and *CAI* were the most distinct markers. Interestingly, the *HIS3* marker

showed statistically significant difference in allelic frequency between commensal, bloodstream and clinical colonizing isolates.

Candida glabrata, unlike other *Candida* species, is unable to undergo dimorphic transition. It is also a commensal member of the human microbiota that can act as an opportunistic pathogen (Fidel et al. 1999). Pfaller et al. (2010) collected 23,305 *Candida* isolates from 133 medical centers in Latin America (15), North America (13), the Asia-Pacific region (21), Africa and the Middle East (8) and Europe (76) between 2001 and 2007. The authors showed that *C. glabrata* accounted for 11.6% of these *Candida* isolates, with a frequency ranging from 7.4% in Latin America to 21.1% in North America. Within the five different geographical regions, the frequency of *C. glabrata* amongst the isolates also varied, ranging from 2.1% (Indonesia) to 34.7% (Australia) in the Asia-Pacific region, from 3.1% (Turkey) to 27.9% (Germany) in Europe, from 7.2% (South Africa) to 14.0% (Saudi Arabia) in Africa and the Middle East and from 3.4% (Mexico) to 11.3% (Brazil) in Latin America.

Pfaller et al. (2010) also studied the susceptibility of all the *Candida* isolates toward fluconazole. In Latin America, fluconazole-resistant isolates increased from 7.1% in 2001 to 14.9% in 2007, while in North America they increased from 13.3% in 2001 to 25.6% in 2004 and then decreased to 13.7% in 2007. In the Asia-Pacific region, fluconazole resistance varied from 24.2% in 2001 to 7.7% in 2004. In Africa and the Middle East, resistance was reported for 30.0% of the isolates in 2003, which decreased to 2.2% in 2007. Europe registered the highest resistance frequencies: 19.3% in 2001 and 19.9% in 2007.

Different genetic fingerprinting methods have been applied to study the diversity of *C. glabrata*: pulsed-field gel electrophoresis (PFGE) [Bennett et al. 2004; Lin et al. 2007; Singh-Babak et al. 2012], Random Amplification of Polymorphic DNA (RAPD) [Paluchowska et al. 2014], multilocus enzyme electrophoresis (MLEE) [De Meeûs et al. 2002], the use of complex DNA probes (Lockhart et al. 1997), multilocus variable-number tandem repeats analysis (MLVA) (Brisse et al. 2009;Abbes et al. 2012) and MLST (Dodgson et al. 2003; Lott et al. 2010).

Dodgson et al. (2003) analyzed 109 *C. glabrata* isolates by MLST typing by using six loci (*FKS*, *LEU2*, *NMT1*,*TRP1*, *UGP1* and *URA3*), which were able to define 30 sequence types. The dendrogram analysis proposed five major groups (I to V); Group III was subdivided into IIIA and IIIB. Europe comprised 37% of the Group I isolates. In the USA, 30% of the isolates were assigned to Group III, while Group IV was not found. Japan over-represented Group IV (54%); Groups I and II were not found in this country.

Katiyar et al. (2016) developed an advanced, robust genetic typing method (PLST, polymorphic locus sequence typing) by targeting the polymorphic tandem repeat-containing loci CgMT-J and CgMT-M. Analysis of these loci resulted in 10 clusters, varying from 2 to 21 strains, among the 104 *C. glabrata* strains. CgMT-J and CgMT-M yielded 24 and 20 alleles, respectively. Diversity index for these two loci were 0.94 and 0.91. Combination of the two loci (i.e. concatenating their sequences and repeating the alignment and phylogenetic analysis) yielded 32 alleles with the diversity index of 0.95. More recently, Carreté et al. (2018) catalogued

single-nucleotide polymorphisms (SNPs) and copy number variations (CNVs) in the 33 globally distributed *C. glabrata* isolates and structured the genetic variation of this species in seven divergent clades (Clades I to VII).

Candida parapsilosis is the second most common *Candida* species isolated from blood cultures in humans. It was isolated for the first time from the stool of a diarrhea patient in Puerto Rico in 1928 by Ashford. This species does not form true hyphae; rather it occurs as yeast or as pseudo hyphae. *C. parapsilosis* was also isolated from non-human sources, like domestic animals, insects, soil and marine environment (Trofa et al. 2008). RAPD (Lehmann et al. 1992), isoenzyme analysis (Lin et al. 1995), DNA-DNA hybridization (Roy and Meyer 1998) and analysis of the nucleotide sequence of the internal transcribed spacers (ITS) [Lin et al. 1995; Kato et al. 2001; Nosek et al. 2002] categorized *C. parapsilosis* into three groups (I, II and III). Later these groups were considered as three different species. Group I retained the *C. parapsilosis* (sensu stricto) name, Group II was named *C. orthopsilosis* and Group III *C. metapsilosis* (Tavanti et al. 2005). Hays et al. (2011) developed a rapid real-time PCR assay, which is able to distinguish these three closely related species. Authors tested 116 clinical isolates and compared the results with the PCR-RFLP patterns of the *SADH* gene as a reference method: 114 isolates were classified as *C. parapsilosis* and 2 as *C. orthopsilosis*; *C. metapsilosis* was not found.

Delfino et al. (2014) studied the fungal pathogens associated with the hands of 129 healthcare workers in Italy. Approximately 39% of these workers tested positive for pathogenic yeasts: *C. parapsilosis* was the most frequent (57%); *C. orthopsilosis* or *C. metapsilosis* isolates were not found.

Four different species-specific polymorphic microsatellite markers (*CP*1, *CP*4, *CP*6 and *B*5) have been widely used to type *C. parapsilosis*. Total 170 independent *C. parapsilosis* strains was isolated from patients' blood samples collected at the Ege University Hospital, Izmir, Turkey, between 2006 and 2014. Microsatellite genotyping of all isolates was achieved. The highest number of distinct alleles was described for *CP6* (31), followed by *CP4* (18), *B5* (17) and *CP1* (7) [Sabino et al. 2015; Hilmioğlu-Polat et al. 2018].

Candida tropicalis is also a diploid yeast. It is mainly isolated from human samples but also from sources like soil, seawater and plants, especially in tropical and subtropical regions. It represents a major cause of nosocomial candidaemia in the elderly worldwide, particularly in the Asia-Pacific region and in Latin America (Scordino et al. 2018). Nucci and Colombo (2007) studied 924 cases of candidaemia in Brazil. They reported that *C. tropicalis* is the causal agent of 21.6% of the cases in adults, 23.2% in elderly patients, 18.5% in children and 11.9% in neonates. MLST analysis was performed with six *C. tropicalis* genes (*ICL1*, *MDR1*, *SAPT2*, *SAPT4*, *XYR1* and ZWF1a): *XYR1* showed the highest typing efficiency, distinguishing three genotypes per polymorphism for 11 polymorphic sites. Nonetheless, MLST database for *C. tropicalis* isolates (https://pubmlst.org/ctropicalis/) is still too small to give an exact estimation of the genetic variation pattern; this database contains 500 sequences obtained from 880 worldwide isolates. The data are arranged in 717 diploid sequence types (DSTs). Out of these, only 16% came from Europe.

Twenty-eight isolates from Italy showed a high degree of genetic homogeneity, as only eight DSTs were obtained (Scordino et al. 2018).

22.1.3 Paracoccidioides

Paracoccidioides brasiliensis and *P. lutzii* are thermodimorphic fungi, which cause paracoccidioidomycosis, a systemic mycosis that affects mainly people in Latin America. They grow as mycelium (the environmental morphotype) at 20–23 °C and as yeast-like multibudding cells in mammal's tissues and in culture at 37 °C. Eighty percent of all cases are reported in Brazil, followed by Venezuela, Colombia, Ecuador, Bolivia and Argentina (Restrepo et al. 2015). *P. brasiliensis comprises four cryptic species: S1 (found in* Southeast and Central-West Brazil and Argentina), *PS2 (*Southeast Brazil and Venezuela*)*, *PS3 (Colombia) and PS4 (*Venezuela*)* [Salgado-Salazar et al. 2010; Teixeira et al. 2013; Camacho and Niño-Vega 2017]. *P. lutzii represents* a monophyletic population found in Central-West Brazil and in Ecuador (Carrero et al. 2008; Teixeira et al. 2013).

Paracoccidioides genetic variability was analyzed by different molecular tools: glycoprotein 43 (GP43) variants' isoelectric points (Moura Campos et al. 1995), *GP43* gene polymorphisms (Cisalpino et al. 1996), RAPD (Calcagno et al. 1998), RFLP (Nino-Vega et al. 2000), electrophoretic karyotyping (Feitosa et al. 2003), microsatellite analysis (Matute et al. 2006) and DNA sequence differences (Matute et al. 2007).

Paracoccidioides genus is composed of five phylogenetic species: four corresponding to *P. brasiliensis* and one to *P. lutzii*. Nuclear gene genealogies support this divergence, but mitochondrial loci do not. To address this contradiction, Turissini et al. (2017) studied 11 previously published gene fragments, 10 newly sequenced nuclear non-coding loci and 10 microsatellites regions. The authors reported that, in spite of sharing the same geographic region, and in some cases the same host, the level of gene flow (interbreeding) was very low among all the described species of *Paracoccidioides*. From this, they concluded that all five genetic groups were well-separated species. They have also revealed that PS3 and PS4 are sister species were closely related to S1. PS2 is the earliest diverged group, thus representing an out-group of PS3/PS4/S1. S1 was described as a monophyletic group.

Desjardins et al. (2011) sequenced and analyzed the genomes of *P. brasiliensis* S1 and PS2 and described a significantly higher percentage of sequence similarity (96%), while similarity to *P. lutzii* was 90%. A total of 176,267 SNPs were identified in PS2 and S1, at the rate of 1 SNP per 132 bases. The comparison of *P. brasiliensis* S1 with *P. lutzii* revealed 501,313 SNPs, at the rate of 1 SNP per 26 bases. Later, Muñoz et al. (2016) analyzed the genome diversity of *P. brasiliensis* PS3 and PS4 lineages. PS3 and PS4 shared 98.4% of genome similarity. These authors have also studied the evolutionary history of *Paracoccidioides*: *P. brasiliensis* and *P. lutzii* diverged about 22.5 million years ago. *P. brasiliensis* lineages were separated about 1.47 million years ago. Within these lineages, PS3 diverged just 23,000 years

ago and S1b 575,000 years ago. The *P. brasiliensis* S1 lineage presented the highest rate of SNPs allele variants.

22.1.4 Histoplasma capsulatum

Histoplasma capsulatum is a heterothallic ascomycete, thermodimorphic fungal species found in filamentous form in the environment. Human and other vertebrates inhale microconidia, macroconidia or fragmented hyphal cells. After reaching the lung alveoli, these undergo rapid conversion into yeast cells, which can disseminate and cause histoplasmosis. Darling (1909) reported the first cases of histoplasmosis in 1905–1906. Immunocompetent individuals can also acquire the disease, but morbidity and mortality rates are higher in immunocompromised populations. *H. capsulatum* presents three distinct taxonomic varieties: *H. capsulatum* var. *capsulatum*, *H. capsulatum* var. *duboisii* and *H. capsulatum* var. *farciminosum*.

The development of histoplasmosis depends on the inhaled inoculum amount and the duration of the exposure, the strain virulence and the host immune response (Deepe and Buesing 2011; Damasceno et al. 2013, 2016). The disease occurs throughout the world, but it is endemic in the Midwestern United States, in the Mississippi and Ohio River valleys, and in Central America (Kauffman 2008). Interestingly, many histoplasmosis cases were reported in the Yangtze River course (Ge et al. 2010; Pan et al. 2013).

Apart from the phenotypic characterization, by antigens and fatty acid profiling, molecular typing methods have also been deployed to study the epidemiology and diversity of *H. capsulatum*, such as probe hybridization, chromosomal DNA typing, RFLP analysis, RAPD analysis and DNA sequencing (de Medeiros Muniz et al. 2010). These authors obtained 51 strains from different regions of Brazil and studied the genetic diversity using M13 PCR fingerprinting, with primers for repetitive DNA sequences, and PCR-RFLP analysis of the internal transcribed spacer 1 (ITS1)-5.8S-ITS2 region of the genome. PCR fingerprinting analysis resulted in three major groups (I, II and III). Group I was divided into subgroups 1A (containing isolates from Rio de Janeiro and from the northeast region of the country) and 1B (isolates from Southeastern Brazil). Group II included isolates from Sao Paulo and Mato Grosso do Sul. Group III included four isolates from the south-eastern, five isolates from the southern and three isolates from the central region. PCR-RFLP amplification of the ITS region of the rDNA gene cluster categorized the 51 isolates in three molecular types (I, II and III). Seventy-four percent of the isolates were gathered in molecular Type I, 5.9% in Type II and 9.6% in Type III. Type I included 38 isolates, 36 of them corresponding to Group I as determined by the PCR fingerprinting method. Type III also corresponded to Group I with some few exceptions.

Rodríguez-Arellanes et al. (2013) determined the diversity of the mating alleles (*MAT1–1* and *MAT1–2*) by PCR in *H. capsulatum* samples from Mexico and Brazil. In Mexico, *MAT1–2* was predominant, accounting for 71.4% of all the isolates. Interestingly, all the isolates from Brazil presented the *MAT1–1* genotype. The

results also revealed that different mating types of *H. capsulatum* were distinctively spread across the American continent. The presence of the two alleles in the same geographical region (Mexico) suggested that the genetic dispersion of the *MAT1* locus could be related to natural reservoirs.

In order to study the genetic diversity at a high-resolution level, different multiallelic microsatellite markers were used: (GA)n, (GT)n and GT(A)n [Carter et al. 1996, 1997, 2001]. These markers distinguished *H. capsulatum* isolates from diverse geographic origins. On the other hand, the (AT)n and (CT) n microsatellite markers failed to amplify the DNA of *H. capsulatum* isolates from the USA; the (CT)n marker could not identify isolates from Australia as well (McEwen et al. 2003).

The microsatellite marker (GA)n sequence allowed the categorization in two major clusters (I and II) of the *H. capsulatum* samples collected from nine distinct bat species from Mexico and Brazil. In Cluster I, the Ia, Ib and Ic subcluster group represented a unique haplotype of *H. capsulatum*; this haplotype was isolated from the migratory bat *Tadarida brasiliensis.* The subclusters Ia and Ib represented highly diversified population in contrast to sub-cluster Ic (Taylor et al. 2012).

MLST analyses of four protein-coding nuclear genes (*ole*, 1-delta-9 fatty acid desaturase; *tub1*, alpha-tubulin; *arf*, ADP ribosylation factor and *H, anti*-H antigen precursor) revealed eight *H. capsulatum* phylogeographic clades. *H. capsulatum* var. *capsulatum* was represented in all the phylogenetic clades, while *H. capsulatum* var. *farciminosum* was identified in North America Class 2, in the Eurasian and in the African clades. The third taxonomic variety, *H. capsulatum* var. *duboisii* was restricted to the African clade (Kasuga et al. 2003). Subsequent studies on *H. capsulatum* samples obtained from cats from California, Colorado, New Mexico and Texas revealed a new clade, mostly related to North America Class I. These studies also suggested that *H. capsulatum* strains isolated from cats are genetically different from strains infecting humans (de Medeiros Muniz et al. 2010; Arunmozhi Balajee et al. 2013).

When compared to the *Coccidioides*, *Blastomyces* and *Paracoccidioides* genera, *Histoplasma* presents higher genetic diversity and worldwide distribution. *Histoplasma* can grow in nitrogen−/phosphate-enriched soil and environments. In this aspect, it can naturally infect avian and chiropteran species, some of them representing long-range migratory reservoirs which contribute to the dispersal of this microorganism (Teixeira et al. 2016).

22.1.5 Sporothrix schenckii

S. schenckii is an ascomycete dimorphic fungus, which infects humans, dogs, cats, rats, armadillos and horses, causing sporotrichosis. The natural habitat of the species is plants, soils and other environmental niches.The first clinical case of cutaneous sporotrichosis was described in Schenck 1898 by Benjamin Schenck. The fungus is found worldwide, but it is more common in the tropical and subtropical areas (Marimon et al. 2006). Mexico, South Africa, India, Japan and South American

countries like Brazil, Peru, Colombia and Uruguay are categorized as endemic regions for the *S. schenckii* infection; Abancay, in the south central Peruvian highlands, is considered as a hyperendemic region (50–60 cases per 100,000 inhabitants per year) [Bustamante and Campos 2001]. Six cryptic species of *S. schenckii* were recognized: *S. brasiliensis*, *S. globosa*, *S. mexicana*, *S. luriei*, *S. pallida* (formerly *S. albicans*) and *S. schenckii* sensu stricto (Marimon et al. 2007). *S. brasiliensis* is the most virulent species, followed by *S. schenckii*; *S. globosa* presents little virulence. There is no report of sporotrichosis caused by *S. pallida*, which is mainly isolated from the environment. The geographical distribution of these species also varies; *S. mexicana* and *S. brasiliensis* are restricted to the respective countries (Arrillaga-Moncrieff et al. 2009; Rodrigues et al. 2013).

Different molecular methods have been employed for studying *S. schenckii* genetic diversity: M13 PCR fingerprinting (Sandhu et al. 1995), PCR targeting mitochondrial DNA (Mora-Cabrera et al. 2001; Marimon et al. 2006), RAPD (Liu et al. 2003), DNA sequencing of rDNA ITS regions (Watanabe et al. 2004), RFLP (Neyra et al. 2005), AFLP (Neyra et al. 2005) and PCR targeting the DNA topoisomerase II gene (Kanbe et al. 2005). Watanabeet al. (2004) targeted the ITS region and the calmodulin gene by the PCR-RFLP technique; four major rDNA types (I to IV) were described, and these types correlated with specific geographical regions. Type I was predominant in Africa and in South and North America, Type II in South America, Type III in North America and Type IV in Australia and Asia.

Rangel-Gamboa et al. (2018) studied the *S. schenckii* population genetics based on the calmodulin and calcium/calmodulin-dependent kinase partial gene sequences in 24 clinical isolates. Eighty-one percent of the Mexican isolates corresponded to *S. schenckii* sensu stricto and 19% to *S. globosa*. Mexican *S. schenckii* sensu stricto isolates were related to the South American ones and displayed a high degree of genetic variation, while the *S. globosa* isolates were related to samples from Asia, Brazil, Spain and the USA and presented only one haplotype.

Espinel-Ingroff et al. (2017) proposed the species-specific Clinical and Laboratory Standards Institute (CLSI) epidemiological cutoff values (ECVs) for *S. schenckii* sensu stricto, *S. brasiliensis*, *S. globosa and S. mexicana* minimal inhibitory concentrations (MICs) for antifungal drugs based on the data from multiple laboratories. For *S. schenckii* and *S. brasiliensis*, species-specific CLSI ECVs were described: for amphotericin B, 4 and 4 μg/ml; itraconazole, 2 and 2 μg/ml; posaconazole, 2 and 2 μg/ml and voriconazole 64 and 32 μg/ml, respectively. Ketoconazole and terbinafine ECVs for *S. brasiliensis* were, respectively, 2 and 0.12 μg/ml, but there was no sufficient data of these drugs for *S. schenckii*. No antifungal drugs MICs data are available for *S. globosa* and *S. mexicana*.

22.1.6 Blastomyces dermatitidis

B. dermatitidis is a thermodimorphic fungus that dwells as saprophyte in environments like moist and sandy soils containing high organic content, in wooded areas and in close proximity to waterways. After the inhalation of microconidia, and the

dimorphic change, it can cause life-threatening blastomycosis in immunocompromised individuals. In immunocompetent ones, symptoms similar to a mild influenza can occur (Yates-Siilata et al. 1995; Saccente and Woods 2010). Blastomycosis, described for the first time by Gilchrist and Stokes in Gilchrist and Stokes 1898, is endemic to Midwestern, Southeastern and South Central United States (Walsh et al. 2003; DiSalvo 1992). In the north-western region of Ontario, Canada, and the north-central American state of Wisconsin, the incidence rates of blastomycosis were reported as 117.2 cases per 100,000 inhabitants (Dwight et al. 2000). It is also endemic to 16 African countries, from Algeria to South Africa (Carman et al. 1989), and cases have been reported in the Middle East and India (DiSalvo 1992), although little is known about *B. dermatitidis* environmental distribution and natural habitat (Randhawa et al. 2013).

Recently, Brown et al. (2018) identified 1392 laboratory-confirmed dimorphic fungal infection cases in Ontario from 1990 to 2015. Blastomycosis was the most common disease (78.4% of the cases), followed by histoplasmosis (15.2%) and coccidioidomycosis (6.4%). On an average, 62 cases were reported a year. Incidence increased from 0.09/100,000 inhabitants in 1995 to 0.52/100,000 in 2001, and then it remained elevated until 2015 (0.48/100,000). As it is also true for paracoccidioidomycosis, men were more susceptible to the infection because estradiol blocks the dimorphic transition. Infection was more common from 40 to 49 years of age.

The first genetic diversity study for *B. dermatitidis* was performed by Fraser et al. (1991) from two different patients' isolates which were shown to be similar by RFLP analysis. Yates-Siilata et al. (1995) described three genotypic groups from RFLP and RAPD data. PCR-RFLP analysis conducted by McCullough et al. (2000) with 59 isolates from North America, India and Africa also resulted in three genotypic groups. Meece et al. (2010) studied 106 isolates from Wisconsin, also by the PCR-RFLP method, and identified two additional types. In this last study, it was possible to reproducibly separate *B. gilchristii*, a cryptic species, from *B. dermatitidis*; furthermore, it was shown that *B. gilchristii* has a very restricted geographical distribution.

One hundred sixty-nine strains of *Blastomyces* spp. were collected by McTaggart et al. (2016) from various regions of North America. The samples were obtained from human, canine and environmental sources, from 1963 to 2013. Population genetics analysis by MLST indicated that *B. dermatitidis* was dispersed from Southeastern United States to Western Canada, while *B. gilchristii* was confined to a northern range. *B. dermatitidis* was categorized into four genetically distinct populations: Population 1 (Nelson River drainage basin), Population 2 (Saint Lawrence River and the northeast portion of the Atlantic Ocean seaboard drainage basins), Population 3 (Gulf of Mexico seaboard and southeast portion of the Atlantic Ocean seaboard drainage basin) and Population 4 (Mississippi River System drainage basin). *B. gilchristii* also represented four distinct populations: Population 1 (Nelson River drainage basin), Population 2 (northern-most tip of the Mississippi River system drainage basin), Population 3 (Saint Lawrence River and northeast Atlantic Ocean seaboard drainage basins) and Population 4 (Eagle River, Oconto Falls and Tomorrow River, in Wisconsin). The authors suggested that the freshwater drainage

basins of North America are mainly responsible for the dispersion, speciation and population structure of *Blastomyces* spp. It was estimated that *B. dermatitidis* and *B. gilchristii* diverged 1.9 million years ago, during the Pleistocene epoch.

22.1.7 Pneumocystis jirovecii

Pneumocystis jirovecii is a yeast-like fungus that causes *Pneumocystis* pneumonia in immunocompromised individuals. *P. jirovecii* was previously known as *P. carinii*, before it was reported as a human-specific pathogen. Nowadays, *P. carinii* specifically refers to the organism that infects rats (Stringer et al. 2002).

Respaldiza et al. (2004) tested 233 healthy Spanish children for the presence of antibodies against *Pneumocystis* epitopes. Results indicated that seroprevalence increased with age (52% at 6 years old, 66% at 10 and 80% at 13) and that the pathogen was widespread in southern Spain.

It is not possible to grow *P. jirovecii* in vitro. Diagnosis was solely dependent on the use of biochemical stains (Gomori methenamine silver, Gram-Weigert or toluidine blue) or of monoclonal antibodies (Kovacs et al. 2001). Molecular methods were developed later to study the epidemiology and diversity of this fungus: DNA sequence analysis, ITS typing of the 18S and the 5.8S rRNA, mitochondrial large subunit ribosomal RNA locus (mtLSU rRNA) and dihydropteroate synthase (DHPS) gene analyses and multi-target PCR-SSCP (single strand conformation polymorphism) [Beard et al. 2004].

By using nested PCR, 20% of 169 healthy subjects' broncho-alveolar lavage (BAL) fluid specimens were found to be colonized with *Pneumocystis* (Nevez et al. 1997). In another study, 20% of oropharyngeal wash samples tested positive for *Pneumocystis* colonization. With the same technique, *Pneumocystis* was detected in 20–43% of BAL specimens from HIV-infected adults (Medrano et al. 2005). The detection of *Pneumocystis* DNA from healthy human samples like BAL, oral wash, induced sputum or nasal-swab samples is normally not possible by non-nested PCR, probably due to very low copy numbers (Wakefield et al. 1990; Leigh et al. 1993; Vargas et al. 2003; Nevez et al. 2006). In Arcenas et al. 2006, Arcenas and collaborators developed a more sensitive real-time PCR detection method.

Curran et al. (2013) studied the genetic diversity of 670 *P. jirovecii* specimens obtained from 476 Northern Ireland individuals from 2008 to 2011. MLST genotyping for ITS1 and mt26S sequences revealed 15 different genotypes and the identification of a strain with a ITS1 allele which was not reported previously.

22.1.8 Penicillium marneffei

Penicillium marneffei is the only dimorphic fungus within the *Penicillium* genus. It causes mycosis in both immunocompetent and immunocompromised hosts. It was firstly isolated from the liver of the bamboo rat (*Rhizomys pruinosus*) in Vietnam (Capponi et al. 1956). The infection in humans was reported in Southeast Asia by

DiSalvo and collaborators in DiSalvo et al. 1973. *P. marneffei* is an important opportunistic pathogen in HIV-infected patients in Northeastern India, Southern China, Thailand, Vietnam, Hong Kong, Cambodia, Malaysia, Taiwan, Laos, Myanmar and Cambodia (Chan et al. 2016). The disease corresponded to the third most common opportunistic infection in HIV-infected persons in Thailand (Supparatpinyo et al. 1994) and to 10% of the infections related to HIV in Hong Kong (Wong et al. 1998). Only four cases of *P. marneffei* infections were reported in non-HIV patients from nonendemic areas (De Monte et al. 2014; Stathakis et al. 2015).

Phylogeny analysis of *P. marneffei* mitochondrial DNA, ITS regions and RNA polymerase II, second largest subunit (rpb2) gene suggested that this fungus is closely related to members of the *Talaromyces* and *Aspergillus* genera (Lau et al. 2017; Tsang et al. 2018).

Vanittanakom et al. (1996) studied the diversity of 22 *P. marneffei* human isolates from Thailand by RFLP. This analysis resulted in the categorization of the isolates in Type I (73%) and Type II (27%). Similar observations have been recorded by Hsueh et al. (2000) with 20 isolates from different Taiwanese patients. Woo et al. (2010) studied *P. marneffei* genetic diversity from the polyketide synthase (PKS) genes polymorphisms. These genes are involved in pigment, toxin and antibiotic production, virulence, aerial hyphae formation and conidiation. Twenty-five PKS genes were found in *P. marneffei* genome, a much higher number than those observed in other pathogenic thermal fungi. The authors suggested that this genomic feature could be involved in the adaptation to different environmental niches, as well as in virulence. Little is known about the acquisition and transmission of the disease caused by *P. marneffei*, since the natural reservoir is unknown; the only known hosts are humans and bamboo rats (Chastain et al. 2017).

MLST genotyping of fragments of the Cox1 mitochondrial gene, and of eight nuclear genes (AbaA, CpeA, NGS, ICL1, LNS, PAA, StlA and MP1), from 24 *P. marneffei* isolates from Hong Kong, Thailand, Vietnam and China was performed by Lasker (2006). Cox1, AbaA, StlA and NGS showed no polymorphisms. CpeA, Icl1, LNS and PAA presented 0.23, 0.69, 0.22 and 0.72% polymorphic nucleotide sites, respectively. In contrast, MP1 presented high polymorphism (21 different nucleotide substitutions). Cluster analysis of five genes (CpeA, Icl1, LNS, PAA and MP1) resulted in 16 different haploid genotypes (HGT) with 11 unique Haploid Sequence Types (HSTs).

22.2 Concluding Remarks

Among approximately 100,000 species of fungi described till date, fewer than 200 cause diseases in humans and other mammals. Among them, yeasts and dimorphic species are clinically relevant for the systemic mycosis they cause, particularly in immunocompromised individuals. These microbes gained attention with the HIV/AIDS pandemia, but nowadays several other clinical conditions such as diabetes, organ transplantation, long hospitalizations and cancer chemotherapy contribute to

the increasing number of patients presenting immune system dysfunctions. Another concern is the growing occurrence of infections in healthy individuals.

The knowledge of genetic diversity, epidemiology and geographic distribution of pathogenic yeasts is essential not only for the treatment but also to the prevention of life-threatening infection burdens. Genetic diversity underlines the phenotypic plasticity associated with environment adaptation, colonization of niches and pathogenicity. Molecular tools such as PCR fingerprinting, RAPD, RFLP and MLST, targeting both mitochondrial and nuclear genes, have allowed the simultaneous assessment of DNA sequence polymorphisms in a large number of distinct environmental and clinical isolates, contributing to phylogenetic, epidemiological and virulence studies. Unlike the invaluable whole-genome approaches, which require heavy funding, sophisticated equipment and bioinformatics software, most of these techniques can now be performed in simpler research facilities and health centers for the proper diagnosis of fungal infections.

Some pathogenic yeast species such as *Cryptococcus neoformans* and *Candida albicans* are found worldwide, while others tend to be restricted to particular regions, such as *Paracoccidioides* spp., *Histoplasma capsulatum*, *Blastomyces dermatitidis* and *Sporothrix schenckii*. The increasing data on the genetic variability of the species have contributed to the understanding of the dispersion dynamics and biological processes involved in host infection. The availability of such information for a largest number of species possibly presents a public health strategy in the present scenario when the multidrug-resistant strains are being detected.

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