Fungal Biology

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Recent Advancement in White Biotechnology Through Fungi

Volume 1: Diversity and Enzymes Perspectives

Fungal Biology

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About the Series

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

More information about this series at http://www.springer.com/series/11224

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Volume 1: Diversity and Enzymes Perspectives

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Foreword

White biotechnology, also known as industrial biotechnology, refers to the use of living cells and/or their enzymes to create industrial products that are more easily degradable, require less energy, create less waste during production and sometimes perform better than the products created using traditional chemical processes. In the twenty-first century, technology was developed to harness fungi to protect human health (through antibiotics, antimicrobial, immunosuppressive agents, value-added products, etc.), which led to industrial-scale production of enzymes, alkaloids, detergents, acids and biosurfactants. During the last decade, considerable progress has been made in white biotechnology research, and further major scientific and technological breakthroughs are expected in the near future. The first large-scale industrial applications of modern biotechnology have been made in the areas of food and animal feed production (agricultural/green biotechnology) and of pharmaceuticals (medical/red biotechnology). In contrast, the production of bioactive compounds through fermentation or enzymatic conversion is known as industrial or white biotechnology. The fungi that are ubiquitous in nature have been isolated from diverse habitats including extreme environments (high temperature, low temperature, salinity, drought, radiation, pressure and pH) and may be associated with plants as epiphytic, endophytic and rhizospheric. Fungal strains are beneficial as well as harmful for human beings. The beneficial fungal strains may play an important role in agriculture, industry and medical sectors. The beneficial fungi play a significance role in plant growth promotion and soil fertility using both direct (solubilization of phosphorus, potassium and zinc; production of indole acetic acid, gibberellic acid, cytokinin and siderophores) and indirect (production of hydrolytic enzymes, siderophores, ammonia, hydrogen cyanides and antibiotics) mechanisms of plant growth promotion for sustainable agriculture. The fungal strains and their products (enzymes, bioactive compounds and secondary metabolites) are very useful for industry, e.g. the discovery of penicillin from *Penicillium chrysogenum* is a milestone in the development of white biotechnology into a fully fledged global industrial technology. Since then, white biotechnology has steadily developed and now plays a key role in several industrial sectors, providing both high-valued nutraceuticals and pharmaceutical products. Fungal

strains and bioactive compounds also play important role in the environmental cleaning.

The present volume on *Recent Advancement in White Biotechnology Through Fungi Vol. 1: Diversity and Enzymes Perspectives* is a very timely publication, which provides state-of-the-art information in the area of white biotechnology, broadly involving fungal-based innovations and applications. This volume comprises 17 chapters. Chapter 1 by Rana et al. describes biodiversity of endophytic fungi from diverse plants, producing wide groups of extracellular hydrolytic enzymes and secondary metabolites for plant growth and soil health, environment bioremediation and bioactive compounds. Chapter 2, presented by Pattnaik and Busi, highlights the interaction of rhizospheric fungi with plants and their potential applications in different fields including agriculture, industrial, pharmaceuticals and biomedical sectors. Chapter 3 by Sharma et al. describes the biodiversity of a ubiquitous fungus, *Trichoderma*, from diverse sources and its applications in the industry as producer of bioactive compounds and extracellular hydrolytic enzymes and in the agriculture as plant growth prompter and biocontrol agents. Chapter 4 by Abdel-Azeem et al. highlights the potential of fungus *Aspergillus*, its biodiversity, ecological significance and industrial applications. In Chapter 5, Pandey et al. describe the mycorrhizal fungi and their biodiversity, ecological significance and industrial applications. Chapter 6 by Abdel-Azeem et al. gives an overview of the studies aimed at the investigation of *Fusarium* biodiversity in a wide variety of different ecological habitats, ecological significances and potential industrial applications. Chapter 7, authored by Naik et al., deals with the new perspectives of industrially important enzymes from endophytic fungi. The enzymes from endophytic fungi have significant potential applications in various industries dealing with chemicals, fuels, food, brewery and wine, animal feed textile, laundry, agriculture, pulp and paper. In Chap. 8, Halder and colleagues emphasize the biosynthesis of fungal chitinolytic enzymes and their potential biotechnological applications in industry and allied sectors. Salwan and Sharma describe the tool for white biotechnology by extremophilic fungal protease production and their applications in Chap. 9. Mandal and Banerjee explain the protease enzymes originating from diverse endophytic fungi and industrial applications in Chap. 10. The most important applications of lipase in pharmaceuticals, pulp and paper, chemicals, textile industries, food processing and biodiesel production have been described by Pérez et al. in Chap. 11. Chapter 12 by Singh et al. describes fungal xylanases, their sources, types and potential biotechnological applications. Susana Rodríguez-Couto presents an overview of fungal laccase, a versatile enzyme for biotechnological applications, in Chap. 13. Karnwal et al. discuss enzymes from different groups of fungi for the textile industry in Chap. 14. Ecological and industrial perspectives of marine fungal white biotechnology are discussed in Chap. 15 by Vala et al. Chapter 16 by Berde et al. highlights the discovery of new extremophilic enzymes from diverse fungal communities and their potential applications in agricultural, industrial, pharmaceutical and allied sectors. Finally, the overall status of fungal white biotechnology is described in Chap. 17 by Meena et al. as the global scenario of fungal white biotechnology in the past, present and future.

Overall, great efforts have been carried out by Dr. Ajar Nath Yadav, his editorial team and scientists from different countries to compile this book as a highly unique, up-to-date source on fungal white biotechnology for students, researchers, scientists and academics. I hope that the readers will find this book highly useful and interesting during their pursuit on fungal biotechnology.

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Dr. H. S. Dhaliwal is presently the vice chancellor of Eternal University, Baru Sahib, Himachal Pradesh, India. Dr. Dhaliwal holds PhD in genetics from the University of California, Riverside, USA (1975). He has 40 years of research, teaching and administrative experience in various capacities. Dr. Dhaliwal is a professor of biotechnology at Eternal University, Baru Sahib, from 2011 to date. He worked as the professor of biotechnology at IIT, Roorkee (2003–2011); founding director of Biotechnology Centre, Punjab Agricultural University, Ludhiana (1992–2003); senior scientist and wheat breeder-cum-director at PAU's Regional Research Station, Gurdaspur (1979–1990); research fellow FMI, Basel Switzerland (1976–1979); and D.F. Jones postdoctoral fellow, University of California, Riverside, USA (1975–1976). Dr. Dhaliwal was elected as fellow, National Academy of Agricultural Sciences, India, (1992); worked as visiting professor, Department of Plant Pathology, Kansas State University, Kansas, USA, (1989); and was a senior research fellow, CIMMYT, Mexico, (1987). He has many national and international awards to his name such as Pesticide India Award from Mycology and Plant Pathology Society of India (1988) and Cash Award from the Federation of Indian Chambers of Commerce and Industry (FICCI) in 1985. He has to his credit more than 400 publications including 250 research papers, 12 reviews, 15 chapters contributed to books, 105 papers presented in meetings, conferences and abstracted, 18 popular articles and 2 books/bulletins/manuals. His important research contributions are the following: identification of new species of wild

diploid wheat *Triticumu rartu* and gathered evidences to implicate *T. urartu* as one of the parents of polyploid wheat; team leader in the development of seven wheat varieties, viz., PBW 54, PBW 120, PBW 138, PBW 175, PBW 222, PBW 226 and PBW 299 approved for cultivation in Punjab and North Western Plain Zone of India; molecular marker-assisted pyramiding of bacterial blight resistance genes *Xa*21 and *Xa*13; and the green revolution semi-dwarfing gene *sd*1 in Dehraduni basmati and developed elite wheat lines biofortified for grain rich in iron and zinc through wide hybridization with related non-progenitor wild wheat species and molecular breeding. Dr. Dhaliwal made a significant contribution to the development of life and epidemiology life cycle of *Tilletia indica* fungus, the causal organism of Karnal bunt disease of wheat and development of Karnal bunt resistance wheat cultivar. Dr. Dhaliwal is a member of several task forces and committees of the Department of Biotechnology, Ministry of Science and Technology, Government of India, New Delhi; chairman, Project Monitoring Committee for Wheat Quality Breeding, Department of Biotechnology, Ministry of Science and Technology, Government of India (2007–2010); chairman of the Project Monitoring Committee in “Agri-biotechnology” of the Department of Biotechnology, Govt. of India, New Delhi (2014–2016); and presently, member of newly constituted Expert Committee for DBT-UDSC Partnership Centre on Genetic Manipulation of Crop Plants at UDSC, New Delhi (2016 onwards).

Preface

White biotechnology, or industrial biotechnology, is drawing much attention as a solution to produce energy, chemicals and other materials from renewable resources. White biotechnology works by marshalling living cells into micro-factories by using biomass as feedstocks. The fungi are used to synthesize functional bioactive compounds, hydrolytic enzymes, and compounds for plant growth promotion and biocontrol agents for the potential biotechnological applications in agriculture, medicine, industry, pharmaceuticals, and allied sectors. White fungal biotechnology is an emerging field in the science arena that supports the revelation of novel and vital biotechnological components. Fungi uses are divided in five major economically important fields: drug manufacturing, food and dietary, environmental, agriculture and biotechnology. The fungi *Aspergillus*, *Bipolaris*, *Cordyceps*, *Fusarium*, *Gaeumannomyces*, *Myceliophthora*, *Penicillium*, *Phoma*, *Piriformospora*, *Pleurotus*, *Trichoderma* and *Xylaria* are highly important fungal groups which can be utilized for production of different antibiotics, enzymes and peptides useful in medical and industrial fields. Secretomic analysis is one of the prominent hubs to identify secretion of enzymes, and the production can be maximized by using genetic engineering approaches in the white biotechnological field.

The present book on *Recent Advancement in White Biotechnology Through Fungi Vol. 1: Diversity and Enzymes Perspectives* covers the biodiversity of diverse groups of fungi reported from extreme environments such as temperature, salinity, drought, radiation, pressure and pH; plant associated as endophytic, epiphytic and rhizospheric; and productions of extracellular enzymes, secondary metabolites and bioactive compounds for diverse processes targeted at therapeutics, diagnostics, bioremediation, agriculture and industries. This book should be immensely useful for the biological sciences, especially to microbiologists, microbial biotechnologists, biochemists, and researchers and scientists of fungal biotechnology. We are honoured that the leading scientists with extensive, in-depth experience and expertise in fungal systems and microbial biotechnology took the time and effort to develop these outstanding chapters. Each chapter is written by internationally recognized researchers/scientists so the reader is given an up-to-date and detailed

account of our knowledge of the white biotechnology and innumerable industrial applications of fungi.

We are indebted to the many people who helped to bring this book to light. The editors wish to thank Mr. Eric Stannard, Senior Editor, Botany, Springer; Dr. Vijai Kumar Gupta and Dr. Maria G. Tuohy, Series Editors, Fungal Biology Springer; and Mr. Rahul Sharma, Project Coordinator, Springer, for the generous assistance, constant support and patience in initializing the volume. Dr. Ajar Nath Yadav gives special thanks to his exquisite wife, Ms. Neelam Yadav, for her constant support and motivations in putting everything together. Dr. Yadav also gives special thanks to his esteemed friends, well-wishers, colleagues and senior faculty members of Eternal University, Baru Sahib, India.

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Chapter 1
Endophytic Fungi: Biodiversity, Ecological Significance, and Potential Industrial Applications

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Abstract Endophytic fungi are abundant and have been reported from all tissues such as roots, stems, leaves, flowers, and fruits. In recent years, research into the beneficial use of endophytic fungi has increased worldwide. In this chapter, we critically review the production of a wide range of secondary metabolites, bioactive compounds from fungal endophytes that are a potential alternative source of secondary plant metabolites and natural producers of high-demand drugs. One of the major areas in endophytic research that holds both economic and environmental potential is bioremediation. During their life span, microbes adapt fast to environmental pollutants and remediate their surrounding microenvironment. In the last two decades, bioremediation has arisen as a suitable alternative for remediating large polluted sites. Endophytic fungi producing ligninolytic enzymes have possible biotechnological applications in lignocellulosic biorefineries. This chapter highlights the recent progress that has been made in screening endophytic fungi for the

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production and commercialization of certain biologically active compounds of fungal endophytic origin.

1.1 Introduction

Microbes such as fungi, bacteria, cyanobacteria, and actinomycetes belonging to a class of plant symbionts residing within plant tissue are referred to as "endophytes" (De Bary 1866). From the germination of seeds to the development of fruits, endophytic microorganisms are associated with different parts of the plant, such as the spermosphere (in seeds), rhizosphere (roots), caulosphere (in stems), phylloplane (in leaves), anthosphere (in flowers), and laimosphere and carposphere (in fruits) (Clay and Holah 1999). To adapt to abiotic and biotic stress factors, endophytic microbes produce bioactive substances (Guo et al. 2008). The associations of endophytic microbes with plants, and in many cases their tolerance to biotic stress factors, have correlated with fungal natural products or biologically active metabolites, such as enzymes, phytohormones, nutrients, and minerals, and also enhance the resistance of the host against herbivores, insects, disease, drought, phytopathogens, and variations in temperature and salinity (Breen 1994; Brem and Leuchtmann 2001; Schulz et al. 2002). Endophytic microbes enhance the resistance of plants to abiotic stress factors such as increasing drought tolerance, high temperature, low temperature, low pH, high salinity, and the presence of heavy metals in the soil (Jalgaonwala et al. 2017). On the other hand, plants provide a protective environment for the growth and multiplication of endophytic microbes, protection from aridness, and longevity via seed transmission to the next generation of host (Khan et al. 2015). One widespread phenomenon in nature is the symbiotic association between fungus and plant.

Initial information about fungal endophytes was found during the year 1904, from endophytes isolated from the seeds of darnel ryegrass (Bezerra et al. 2012; Freeman 1904). Endophytic fungi are a diverse and useful group of microorganisms reported to colonize plants in different parts of world, such as the Arctic (Fisher et al. 1995) and Antarctic (Rosa et al. 2009), and in geothermal lands (Redman et al. 2002), deserts (Bashyal et al. 2005), oceans (Wang et al. 2006b), rainforests (Strobel 2002), mangrove swamps (Lin et al. 2008b), and coastal forests (Suryanarayanan et al. 2005). Various secondary metabolites, for instance, alkaloids, cyclohexanes, flavonoids, hydrocarbons, quinines, and terpenes, have been reported to be synthesized by fungal endophytes and have various biological properties including antimicrobial, antioxidant, antidiabetic, anticancer, antihypercholesterolemic, and antiproliferative activities and cytotoxicity, and they are used in biofuel manufacturing (Fernandes et al. 2015; Naik and Krishnamurthy 2010; Ruma et al. 2013). Endophytic fungi produce various kinds of extracellular enzymes, i.e., hydrolases, lyases, oxidoreductases, and (Traving et al. 2015). In another study, endophytic microbes producing enzymes could help to initiate the symbiotic process (Hallmann et al. 1997). Fungal endophytes have been reported to produce hydrolytic enzymes

such as cellulase, lipoidase, pectinase, proteinase, and phenol oxidase so as to overcome the defense response against the host (Krishnamurthy and Naik 2017; Naik et al. 2009; Oses et al. 2006). Various organic compounds, for instance, cellulose, glucose, hemicelluloses, keratin, lignin, lipids, oligosaccharides, pectin, and proteins, have been reported to be degraded by the endophytic fungi (Kudanga and Mwenje 2005; Tomita 2003). Endophytic microbes have been reported in almost all plant studies (Suman et al. 2016; Verma et al. 2013, 2014a, 2015a). This chapter describes the biodiversity of endophytic fungi from diverse plants, producing wide groups of extracellular hydrolytic enzymes, bioactive compounds, and secondary metabolites useful for plant growth and soil health for sustainable agriculture, for environment bioremediation, and for different processes in industry.

1.2 Biodiversity and Distribution of Fungal Endophytes

Recently, a greater progress has been made in fungal endophytic research. Fungal endophytes have been found to colonize land plants everywhere on earth. They have been isolated from boreal forests, tropical climates, diverse xeric environments, extreme arctic environments, ferns, gymnosperms, and angiosperms (Mohali et al. 2005; Selim et al. 2017; Šraj-Kržič et al. 2006; Suryanarayanan et al. 2000). Endophytic fungi play an important role in protecting their host from attack by phytopathogens and also facilitate the solubilization of the macronutrients phosphorus, potassium, and zinc; the fixation of atmospheric nitrogen; and the production of various hydrolytic enzymes, ammonia, siderophore, and hydrogen cyanide (HCN) (Maheshwari 2011; Rana et al. 2016a, b, 2017; Verma et al. 2015b, c, 2016a, b).

From a review of the diverse research on endophytic fungi diversity, it can be concluded that reported fungi belong to diverse phyla including Ascomycota, Basidiomycota, and Mucoromycota (Fig. 1.1a). Figure 1.1b presents the biodiversity and abundance of endophytic fungi reported from chick pea, common pea, maize, pigeon pea, rice, soybean, tomato, and wheat. Figure 1.1c presents the relative distribution and biodiversity of endophytic fungi reported from different host plants, showing the common and host-specific endophytic fungi. Figure 1.1d is a Venn diagram showing the endophytic fungal diversity of leguminous and nonleguminous crops. There are many reports of the microbiomes as niche-specific diversity caused by diverse environmental conditions, including low temperature (Yadav 2015; Yadav et al. 2015a, b, 2016, 2017c), high temperature (Kumar et al. 2014; Sahay et al. 2017), salinity (Yadav et al. 2015c, 2018a), drought (Verma et al. 2014a, 2016b), pH (Verma et al. 2013), and multiple extreme conditions (Saxena et al. 2016; Verma et al. 2017; Yadav et al. 2015c, 2018b). Suman et al. (2016) reported niche-specific endophytic microbes from 17 different host plants. Table 1.1 presents the biodiversity of endophytic fungi reported from these diverse host plants.

Impullitti and Malvick (2013) reported fungal endophytes such as *Alternaria* sp., *Cladosporium* sp., *Davidella* sp., *Diaporthe* sp., *Epicoccum* sp., *Fusarium* sp., *Phialophora* sp., *Phoma* sp., *Phomopsis* sp., *Plectosphaerella* sp., *Trichoderma* sp.,

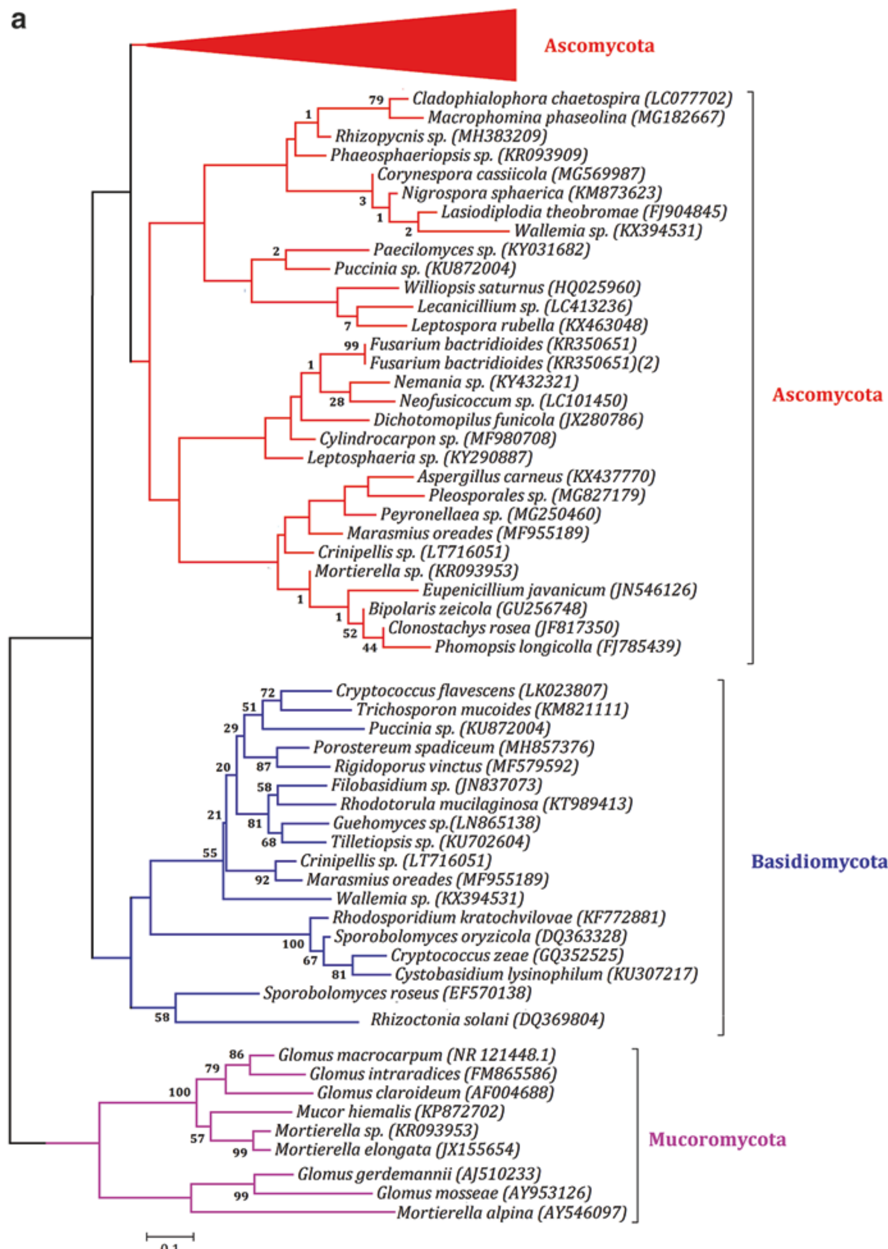

Fig. 1.1 (**a**) Phylogenetic tree shows the relationship among different groups of endophytic fungi isolated from different host plants. (**b**) Abundance of endophytic fungi belonging to diverse phyla isolated from different host plants. (**c**) Diversity and distribution of endophytic fungi of different crops. (**d**) Venn diagram showing niche-specific microbes reported from leguminous and nonleguminous crops. Wheat *(Triticum aestivum)*: (Colla et al. 2015; Comby et al. 2017; Fisher and Petrini 1992; Keyser et al. 2016; Köhl et al. 2015; Larran et al. 2002, 2007, 2018; Ofek-Lalzar et al. 2016; Sieber et al. 1988; Spagnoletti et al. 2017; Wakelin et al. 2004); rice (*Oryza sativa*): (Naik et al. 2009;

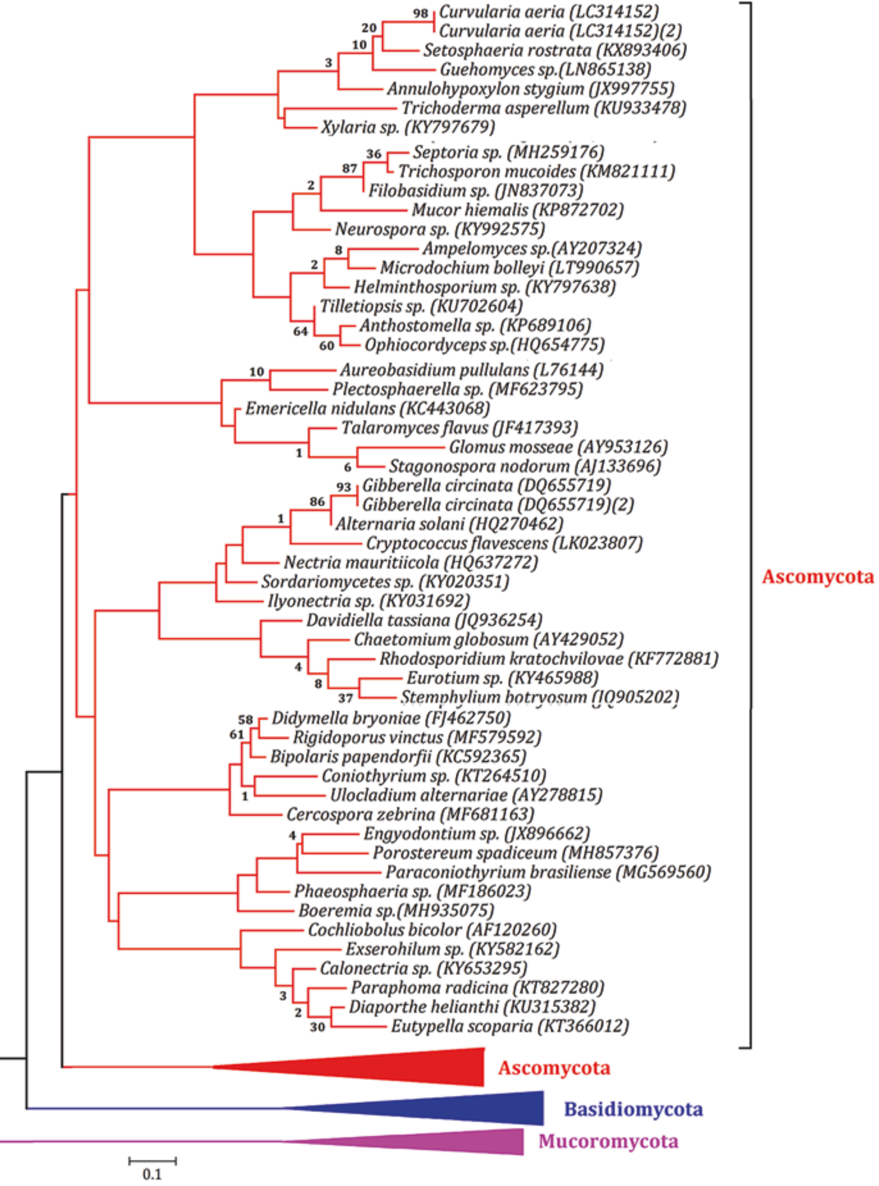

Fig. 1.1 (continued) Potshangbam et al. 2017; Tian et al. 2004; Wang et al. 2016; Yuan et al. 2010); tomato (*Solanum lycopersicum)*: (Bogner et al. 2016; Chadha et al. 2015; Larran et al. 2001; Tian et al. 2014); maize (*Zea mays*): (Amin 2013; Köhl et al. 2015; Nassar et al. 2005; Pan et al. 2008; Potshangbam et al. 2017; Renuka and Ramanujam 2016; Saunders and Kohn 2008; Xing et al. 2018); chickpea (*Cicer arietinum)*: (Narayan et al. 2017; Singh and Gaur 2017); soybean *(Glycine max)*: (de Souza Leite et al. 2013; Fernandes et al. 2015; Hamayun et al. 2017; Impullitti and Malvick 2013; Khan et al. 2011b, 2012b; Rothen et al. 2017; Tenguria and Firodiya 2013; Yang et al. 2014, 2018; Zhao et al. 2018); common bean (*Phaseolus vulgaris)*: (dos Santos et al. 2016; Gonzaga et al. 2015; Marcenaro and Valkonen 2016; Parsa et al. 2016; Pierre et al. 2016); pigeon pea (*Cajanus cajan)*: (Gao et al. 2011, 2012; Zhao et al. 2012, 2013, 2014)

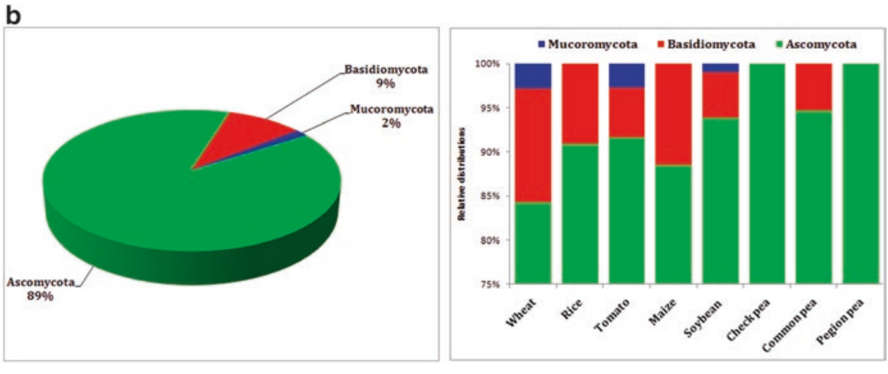

Fig. 1.1 (continued)

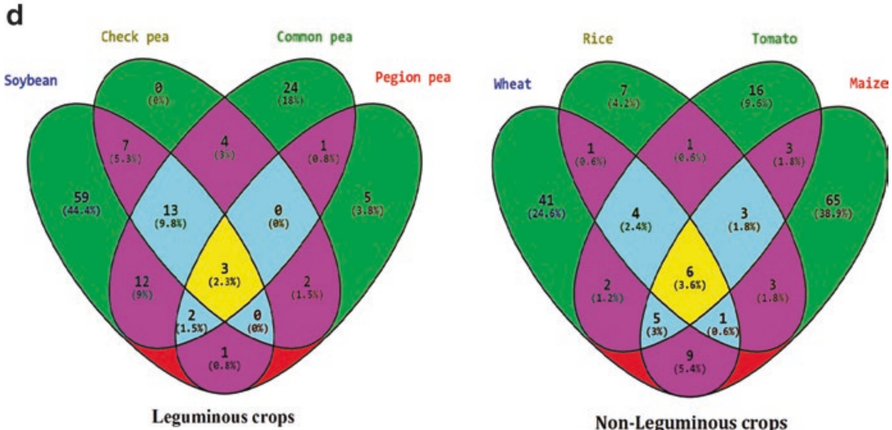

Fig. 1.1 (continued)

and *Verticillium* sp. in soybean plants; these were found by using culture-dependent and culture-independent methods. Tenguria and Firodiya (2013) isolated endophytic fungi, including *Acremonium* sp., *Alternaria alternate*, *Aspergillus* sp., *Colletotrichum* sp., *Emericella nidulans*, *Fusarium* sp., *Penicillium* sp., and *Phoma* sp. from leaves of fresh *Glycine max* collected from the central region of Madhya Pradesh, India. Fernandes et al. (2015) reported the diversity of fungal endophytes in the leaves and roots of *G. max* (dos Santos Souza and dos Santos 2017). In that study, *Ampelomyces* sp., *Cladosporium cladosporioides*, *Colletotrichum gloeosporioides*, *Diaporthe helianthi*, *Guignardia mangiferae*, and *Phoma* sp. were isolated from the leaves, and the dominance of *Fusarium oxysporum*, *Fusarium solani*, and *Fusarium* sp. was greater in the roots (Fernandes et al. 2015). Hamayun et al. (2017) reported *Porostereum spadiceum* AGH786 as a novel gibberellin (GA)-synthesizing fungal endophyte that promoted the growth of soybeans and was capable of producing six types of GAs (Onofre et al. 2013).

Larran et al. (2007) isolated *Alternaria alternata*, *Cladosporium herbarum*, *Epicoccum nigrum*, *Cryptococcus* sp., *Rhodotorula rubra*, *Penicillium* sp., and *Fusarium graminearum* with the highest colonization frequency from wheat (dos Santos Souza and dos Santos 2017). Amin (2013) isolated *Acremonium* sp., *Aspergillus* sp., *Botryodiplodia* sp., *Fusarium* sp., *Penicillium* sp., and *Trichoderma* sp. from the roots of *Zea mays* (Azevedo et al. 2000). Chadha et al. (2015) isolated endophytic fungi identified as *Aspergillus niger*, *Aspergillus* sp., *A. versicolor*, *Chaetomium globosum*, *Fusarium fusarioides*, *F. moniliforme*, *F. oxysporum*, *F. semitectum*, *F. solani*, *Mucor hiemalis*, *Mucor* sp., and *Trichoderma pseudokoningii* from the roots of tomato, and further screened for different plant growth-promoting attributes. All the isolates showed that they were capable of solubilizing phosphorus, 7 showed siderophore production, 4produced HCN, and 3 produced ammonia. The production of indole acetic acid (IAA) was found to be highest in *Fusarium fusarioides.* Renuka and Ramanujam (2016) determined *Acremonium zeae*, *Coprinopsis cinerea*, *Fusarium fujikuroi*, *Gibberella moniliformis*, *Nemania* sp.,

Table 1.1 Biodiversity of endophytic fungi isolated from diverse host plants worldwide

Host plant	Endophytic fungi	Reference
Thuja plicata	*Xylaria*	Adnan et al. (2018)
Eremophila longifolia, *Eremophila maculata*	*Alternaria*, *Preussia*	Zaferanloo et al. (2018)
Oxalis corniculata	*Aspergillus*, *Fusarium*	Bilal et al. (2018)
Populus trichocarpa	*Cladosporium*, *Penicillium*, *Trichoderma*	Huang et al. (2018)
	Alternaria, *Aspergillus*, *Boeremia*, *Chaetomium*, *Chaetosphaeronema*, *Cladosporium*, *Curvularia*, *Fusarium*, *Paecilomyces*, *Penicillium*, *Periconia*, *Phoma*, *Phyllosticta*, *Pleosporales, Preussia*, *Pseudodiplodia*, *Pseudopithomyces*, *Purpureocillium*, *Rhizopus*, *Schizothecium*, *Talaromyces*, *Trichoderma*, *Truncatella*	Pieterse et al. (2018)
Calotropis procera	*Acremonium*, *Acremonium*, *Cercospora*, *Cladosporium*, *Colletotrichum*, *Curvularia*, *Diplodina*, *Glomerella*, *Microascus*, *Phaeoramularia*, *Rhodotorula*, *Xylaria*	Nascimento et al. (2015)
Pinus wallichiana	*Alternaria*, *Anthostomella*, *Aspergillus*, *Cadophora*, *Cladosporium*, *Cochliobolus*, *Coniochaeta*, *Coniothyrium*, *Epicoccum*, *Fimetariella*, *Fusarium*, *Geopyxis*, *Lecythophora*, *Leptosphaeria*, *Lophiostoma*, *Lophodermium*, *Microdiplodia*, *Neurospora*, *Nigrospora*, *Paraconiothyrium*, *Penicillium*, *Pestalotiopsis*, *Phoma*, *Phomopsis*, *Preussia*, *Pseudoplectania*, *Rachicladosporium*, *Rosellinia*, *Sclerostagonospora*, *Sordaria*, *Sporormiella*, *Therrya*, *Tricharina*, *Trichoderma*, *Thielavia*, *Tritirachium*, *Truncatella*, *Xylaria*	Qadri et al. (2014)
Brassica napus	*Acremonium*, *Alternaria*, *Arthrinium*, *Aspergillus*, *Aureobasidium*, *Botrytis*, *Chaetomium*, *Clonostachys*, *Cryptococcus*, *Dioszegia*, *Dothidea*, *Dothiorella*, *Epicoccum*, *Fusarium*, *Guignardia*, *Hypoxylon*, *Leptosphaeria*, *Macrophomina*, *Nigrospora*, *Penicillium*, *Periconia*, *Phoma*, *Rhizoctonia*, *Rhizopus*, *Simplicillium*, *Sporidiobolus*, *Sporobolomyces*	Zhang et al. (2014)
Taxus x media	*Alternaria*, *Colletotrichum*, *Gibberella*, *Glomerella*, *Guignardia*, *Nigrospora*, *Phoma*, *Phomopsis*	Xiong et al. (2013)
Stellera chamaejasme	*Acremonium*, *Alternaria*, *Aporospora*, *Ascochyta*, *Aspergillus*, *Bionectria*, *Botryotinia*, *Cadophora*, *Colletotrichum*, *Dothiorella*, *Emericellopsis*, *Eucasphaeria*, *Eupenicillium*, *Fusarium*, *Geomyces*, *Ilyonectria*, *Leptosphaeria*, *Mucor*, *Nectria*, *Neonectria*, *Paecilomyces*, *Paraphoma*, *Penicillium*, *Schizophyllum*, *Scytalidium*, *Sordaria*, *Sporormiella*	Jin et al. (2013)
Panax ginseng	*Aspergillus*, *Cladosporium*, *Engyodontium*, *Fusarium*, *Penicillium*, *Plectosphaerella*, *Verticillium*	Wu et al. (2013)

Kigelia africana	*Alternaria*, *Aspergillus*, *Botryodiplodia*, *Chaetomium*, *Colletotrichum*, *Curvularia*, *Drechslera*, *Fusarium*, *Mucor*, *Nigrospora*, *Nodulisporium*, *Penicillium*, *Pestalotiopsis*, *Phoma*, *Phomopsis*, *Rhizopus*, *Trichoderma*	Maheswari and Rajagopal (2013)
Jatropha curcas	*Alternaria*, *Chaetomium*, *Colletotrichum*, *Fusarium*, *Guignardia*, *Nigrospora*	Kumar and Kaushik (2013)
Glycine max	*Alternaria*, *Ampelomyces*, *Annulohypoxylon*, *Arthrinium*, *Cercospora*, *Chaetomium*, *Cladosporium*, *Cochliobolus*, *Colletotrichum*, *Curvularia*, *Davidiella*, *Diaporthe*, *Didymella*, *Epicoccum*, *Eutypella*, *Fusarium*, *Gibberella*, *Guignardia*, *Leptospora*, *Magnaporthe*, *Myrothecium*, *Nectria*, *Neofusicoccum*, *Nigrospora*, *Ophiognomonia*, *Paraconiothyrium*, *Phaeosphaeriopsis*, *Phoma*, *Phomopsis*, *Rhodotorula*, *Sporobolomyces*, *Stemphylium*, *Xylaria*	de Souza Leite et al. (2013)
Cannabis sativa	*Aspergillus*, *Chaetomium*, *Eupenicillium*, *Penicillium*	Kusari P et al. (2013a)
Vitis vinifera	*Absidia*, *Alternaria*, *Aspergillus*, *Aureobasidium*, *Botrytis*, *Cladosporium*, *Epicoccum*, *Fusarium*, *Mortierella*, *Mucor*, *Penicillium*, *Pithomyces*, *Rhizopus*, *Trichoderma*, *Umbelopsis*, *Zygorhynchus*	Pancher et al. (2012)
Trichilia elegans	*Cordyceps*, *Diaporthe*, *Phomopsis*	Rhoden et al. (2012)
Tinospora sinensis	*Acremonium*, *Alternaria*, *Aspergillus*, *Botryosphaeria*, *Botrytis*, *Cladosporium*, *Chaetomium*, *Colletotrichum*, *Curvularia*, *Drechslera*, *Emericella*, *Fusarium*, *Guignardia*, *Humicola*, *Monilia*, *Nigrospora*, *Penicillium*, *Pseudofusicoccum*, *Trichoderma*, *Veronaea*	Mishra et al. (2012)
Stryphnodendron adstringens	*Alternaria*, *Arthrobotrys*, *Aspergillus*, *Botryosphaeria*, *Cladosporium*, *Colletotrichum*, *Coniochaeta*, *Cytospora*, *Diaporthe*, *Guignardia*, *Fimetariella*, *Massarina*, *Muscodor*, *Neofusicoccum*, *Nigrospora*, *Paraconiothyrium*, *Penicillium*, *Pestalotiopsis*, *Phomopsis*, *Preussia*, *Pseudofusicoccum*, *Sordaria*, *Sporormiella*, *Trichoderma*, *Xylaria*	Carvalho et al. (2012)
Sapindus saponaria	*Alternaria*, *Cochliobolus*, *Curvularia*, *Diaporthe*, *Phoma*, *Phomopsis*	García et al. (2012)
Reynoutria japonica	*Alternaria*, *Arthrinium*, *Bionectria*, *Colletotrichum*, *Didymella*, *Glomerella*, *Nigrospora*, *Pestalotiopsis*, *Phoma*, *Phomopsis*, *Phyllosticta*, *Septoria*, *Xylaria*	Kurose et al. (2012)
Piper hispidum	*Alternaria*, *Bipolaris*, *Colletotrichum*, *Glomerella*, *Guignardia*, *Lasiodiplodia*, *Marasmius*, *Phlebia*, *Phoma*, *Phomopsis*, *Schizophyllum*	Orlandelli et al. (2012)
Picea abies	*Acephala*, *Chalara*, *Cistella*, *Cladosporium*, *Entomocorticium*, *Fomitopsis*, *Lophodermium*, *Mollisia*, *Mycena*, *Neonectria*, *Ophiostoma*, *Phacidiopycnis*, *Phacidium*, *Phialocephala*, *Rhizoscyphus*, *Rhizosphaera*, *Sarea*, *Scleroconidioma*, *Sirococcus*, *Valsa*, *Xylomelasma*, *Zalerion*	Koukol et al. (2012)

(continued)

Table 1.1 (continued)

Host plant	Endophytic fungi	Reference
Opuntia ficus-indica	*Acremonium*, *Aspergillus*, *Cladosporium*, *Fusarium*, *Monodictys*, *Nigrospora*, *Penicillium*, *Pestalotiopsis*, *Phoma*, *Phomopsis*, *Tetraploa*, *Xylaria*	Bezerra et al. (2012)
Nyctanthes arbor-tristis	*Acremonium*, *Alternaria*, *Aspergillus*, *Chaetomium*, *Cladosporium*, *Colletotrichum*, *Drechslera*, *Humicola*, *Fusarium*, *Nigrospora*, *Penicillium*, *Phomopsis*, *Rhizoctonia*	Gond et al. (2012)
Ginkgo biloba	*Alternaria*, *Cladosporium*, *Colletotrichum*, *Fusarium*, *Pestalotiopsis*, *Peyronellaea*, *Phoma*, *Phomopsis*, *Phyllosticta*	Thongsandee et al. (2012)
Echinacea purpurea	*Ceratobasidium*, *Cladosporium*, *Colletotrichum*, *Fusarium*, *Glomerella*, *Mycoleptodiscus*	Rosa et al. (2012)
Cinnamomum camphora	*Alternaria*, *Arthrinium*, *Arthrobotrys*, *Aspergillus*, *Chaetomium*, *Chaetophoma*, *Cladosporium*, *Curvularia*, *Drechslera*, *Gliomastix*, *Humicola*, *Nigrospora*, *Penicillium*, *Periconia*, *Pestalotiopsis*, *Phacidium*, *Phomopsis*, *Phyllosticta*, *Stachybotrys*, *Trichoderma*	Kharwar et al. (2012)
Acer tataricum subsp. *ginnala*	*Alternaria*, *Cladosporium*, *Epicoccum*, *Fusarium*, *Neurospora*, *Penicillium*, *Phoma*, *Phomopsis*, *Trichoderma*	Qi et al. (2012)
Tylophora indica	*Alternaria*, *Chaetomium*, *Colletotrichum*, *Nigrospora*, *Thielavia*	Tamura et al. (2011)
Taxus globosa	*Alternaria*, *Aspergillus*, *Annulohypoxylon*, *Cercophora*, *Cochliobolus*, *Colletotrichum*, *Conoplea*, *Coprinellus*, *Daldinia*, *Hypocrea*, *Hypoxylon*, *Lecythophora*, *Letendraea*, *Massarina*, *Nigrospora*, *Penicillium*, *Phialophorophoma*, *Phoma*, *Polyporus*, *Sporormia*, *Trametes*, *Trichophaea*, *Xylaria*, *Xylomelasma*	Rivera-Orduña et al. (2011)
Solanum cernuum	*Arthrobotrys*, *Bipolaris*, *Botryosphaeria*, *Candida*, *Cercospora*, *Colletotrichum*, *Coprinellus*, *Cryptococcus*, *Curvularia*, *Diatrypella*, *Edenia*, *Eutypella*, *Fusarium*, *Glomerella*, *Leptosphaeria*, *Mucor*, *Petriella*, *Phoma*, *Meyerozyma*, *Flavodon*, *Hapalopilus*, *Hohenbuehelia*, *Kwoniella*, *Oudemansiella*, *Phanerochaete*, *Phlebia*, *Phlebiopsis*, *Schizophyllum*	Vieira et al. (2011)
Lippia sidoides	*Alternaria*, *Colletotrichum*, *Corynespora*, *Curvularia*, *Drechslera*, *Fusarium*, *Guignardia*, *Microascus*, *Paecilomyces*, *Periconia*, *Phoma*, *Phomopsis*	de Siqueira et al. (2011)
Ledum palustre	*Arthrinium*, *Fusarium*, *Lecythophora*, *Penicillium*, *Sordaria*, *Sphaeriothyrium*	Tejesvi et al. (2011)
Dendrobium thyrsiflorum	*Alternaria*, *Colletotrichum*, *Epicoccum*, *Fusarium*, *Glomerella*, *Leptosphaerulina*, *Pestalotiopsis*, *Phoma*, *Rhizopus*, *Xylaria*	Xing et al. (2011)
Dendrobium devonianum	*Acremonium*, *Arthrinium*, *Cladosporium*, *Fusarium*, *Glomerella*, *Leptosphaerulina*, *Phoma*, *Pestalotiopsis*, *Rhizopus*, *Trichoderma*, *Xylaria*	Xing et al. (2011)

Aquilaria sinensis	*Chaetomium*, *Cladosporium*, *Coniothyrium*, *Epicoccum*, *Fusarium*, *Hypocrea*, *Lasiodiplodia*, *Leptosphaerulina*, *Paraconiothyrium*, *Phaeoacremonium*, *Phoma*, *Pichia*, *Rhizomucor*, *Xylaria*	Cui et al. (2011)
Theobroma cacao	*Acremonium*, *Arthrinium*, *Aspergillus*, *Clonostachys*, *Colletotrichum*, *Coniothyrium*, *Curvularia*, *Cylindrocladium*, *Fusarium*, *Gliocladium*, *Lasiodiplodia*, *Myrothecium*, *Paecilomyces*, *Penicillium*, *Pestalotiopsis*, *Phoma*, *Septoria*, *Talaromyces*, *Tolypocladium*, *Trichoderma*, *Verticillium*	Hanada et al. (2010)
Dendrobium loddigesii	*Acremonium*, *Alternaria*, *Ampelomyces*, *Bionectria*, *Cercophora*, *Chaetomella*, *Cladosporium*, *Colletotrichum*, *Davidiella*, *Fusarium*, *Lasiodiplodia*, *Nigrospora*, *Paraconiothyrium*, *Pyrenochaeta*, *Sirodesmium*, *Verticillium*, *Xylaria*	Chen et al. (2010)
Colobanthus quitensis	*Aspergillus*, *Cadophora*, *Davidiella*, *Entrophospora*, *Fusarium*, *Geomyces*, *Gyoerffyella*, *Microdochium*, *Mycocentrospora*, *Phaeosphaeria*	Rosa et al. (2010)
Dracaena cambodiana, *Aquilaria sinensis*	*Botryosphaeria*, *Calcarisporium*, *Cephalosporium*, *Colletotrichum*, *Fusarium*, *Geotrichum*, *Gonytrichum*, *Guignardia*, *Mortierella*, *Rhinocladiella*, *Mycelia*, *Pleospora*	Gong and Guo (2009)
Artemisia	*Alternaria*, *Colletotrichum*, *Phomopsis*, *Xylaria*	Huang et al. (2009)
Medicinal plants	*Alternaria*, *Colletotrichum*, *Phoma*, *Phomopsis*, *Xylariales*	Huang et al. (2008)
Aegle marmelos	*Alternaria*, *Aspergillus*, *Chaetomium*, *Curvularia*, *Drechslera*, *Emericella*, *Fusarium*, *Nigrospora*, *Rhizoctonia*, *Stenella*	Gond et al. (2007)

Penicillium sp., *Cladosporium oxysporum*, *Rigidoporus vinctus*, *Colletotrichum boninense*, *Sarocladium zeae*, *Epicoccum sorghinum*, *Curvularia lunata*, *Scopulariopsis gracilis*, and *Colletotrichum gloeosporioides* from the leaf, stem, and root fragments of different varieties of maize. Wang et al. (2016) isolated endophytic fungal and bacterial strains from sprouts, stems, and roots simultaneously in rice plants. *Aspergillus*, *Cryptococcus*, *Eurotium*, *Fusarium*, *Penicillium*, *Septoria*, and *Wallemia* were the most frequently detected genera in rice plants. The dominant fungal genera, including *Aspergillus*, *Penicillium*, and *Trichosporon*, coexisted in the stems and roots. Furthermore, *Cryptococcus*, *Fusarium, Penicillium*, *Pestalotiopsis*, and *Verticillium* were detected in the sprouts, stems, and roots simultaneously. Xing et al. (2018) isolated *Alternaria alternata*, *Aspergillus flavus*, *A. niger*, *Bipolaris zeicola*, *Chaetomium murorum*, *Cladosporium sphaerospermum*, *Fusarium proliferatum*, *F. verticillioides*, *Penicillium aurantiogriseum*, *P. oxalicum*, *P. polonicum*, *Sarocladium zeae*, and *Trichoderma gamsii* from maize seeds.

1.3 Biotechnological Applications of Endophytic Fungi

Over the past several decades, endophytic fungi separated from numerous plant sources have been recognized as valuable sources of natural products for agronomy, industry, and biomedical development, and also produce extracellular hydrolase enzymes, such as pectinases, cellulases, lipases, amylases, laccases, xylanase, and proteases, as one of the resistance mechanisms against pathogenic organisms and for gaining nutrition from the host. From medicinal plants, endophytic fungi synthesizing hydrolytic enzymes have been reported (Khan et al. 2017; Saxena et al. 2015a; Sunitha et al. 2013; Yadav et al. 2012). Extracellular enzymes target various macromolecules, e.g., lignin, proteins, carbohydrates, sugar-based polymers, to break them down into simpler ones. The production of extracellular enzymes has been measured qualitatively and quantitatively, from using agar plate-based to applying advanced spectrophotometric methods (Yadav et al. 2017a, b).

1.3.1 Bioresources of Hydrolytic Enzymes

Endophytic microorganisms are well known, as they spend the whole of their life cycle inhabiting the inside of tissues in host plants without causing them any obvious harm (Bezerra et al. 2012; Kaul et al. 2013; Tan and Zou 2001; Yadav et al. 2016). The endophytic microbes guard their host plants against attack by other microbes, insects, and herbivore animals, furthermore providing other benefits, for instance, the production of numerous plant growth regulators, enzymes, and other chemical compounds (Azevedo et al. 2000; Bezerra et al. 2012). In addition, these endophytic microbes have also been reported to produce diverse metabolites, including alkaloids, flavonoids, isocoumarin derivatives, peptides, phenolic acids,

phenols, quinones, steroids, and terpenoids (Rana et al. 2016b; Yadav et al. 2015). In recent times, fungal endophytes have become responsive, as they are an appropriate reserve for the degradation of polycyclic aromatic hydrocarbons, which are well known as a toxic class of environmental contaminants (Bezerra et al. 2012; Dai et al. 2010). Additionally, endophytes are also known for the production of various extracellular enzymes, such as cellulases, esterases, lipases, pectinases, proteases, and xylanases, which play an important role in protecting themselves from the defense response of the host or in attaining nourishment from the soil (Bezerra et al. 2012; Suto et al. 2002). Therefore, endophytes are an enormous source of naturally active products that are of marked significance to the agricultural, industrial, and medical sectors (Hazalin et al. 2012). The major industries that utilize microbial enzymes include biomaterials, cellulose, cosmetics, detergents, energy, fine chemicals, food, leather, paper, pharmaceuticals, and textiles, (Bezerra et al. 2012; Suto et al. 2002; Yadav et al. 2015). Table 1.2 shows the diversity and abundance of diverse extracellular hydrolytic enzyme production by different groups of endophytic fungi reported from diverse host plants worldwide.

1.3.1.1 Cellulases

Cellulases are basically the enzymes that catalyze cellulolysis, which involves the degradation of the cellulose and certain related polysaccharides. Certain bacteria, fungi, and protozoans are known to synthesize the enzyme (Singh 2006). Different types of cellulases are known that differ from each other structurally and mechanistically, and these include endocellulases, exocellulases, also known as cellobiohydrolases, cellobiases or beta-glucosidases, oxidative cellulases, cellulose phosphorylases. Cellulases from microbes find diverse applications such as use with a supplement of hemicellulases, pectinases, ligninases, and associated enzymes (Adav and Sze 2014). In addition to lignocellulosic bioenergy, cellulase are important in the agricultural, animal feed, brewing, food, laundry, paper and pulp, textile, and wine industries (Adav and Sze 2014; Bhat and Bhat 1997; Mandels 1985; Ryu and Mandels 1980). The most commonly studied cellulolytic fungi include the species of *Aspergillus*, *Humicola*, *Penicillium*, and *Trichoderma* (Sukumaran et al. 2005).

Peng and Chen (2007), obtained 141 isolates of fungal endophytes from the stems of seven oleaginous plant species. These isolates belonged to genera including *Cephalosporium*, *Microsphaeropsis*, *Nigrospora*, *Phomopsis*, and *Sclerocystis*. The oil content of these isolates ranged from 21.3% to 35.0% of dry cell weight. Further, the strains also produced cellulase in addition to microbial oil when cultured on solid-state medium consisting of steam-exploded wheat straw, wheat bran, and water. The yield of cellulase ranged from 0.31 to 0.69 filter paper unit per gram of initial dry substrate. Bezerra et al. (2012) isolated 44 isolates of fungal endophytes from *Opuntia ficus-indica* and assessed their ability to synthesize hydrolytic enzymes such as cellulases, pectinases, proteases, and xylanases. The cellulase producers were identified as *Acremonium terricola*, *Aspergillus japonicas*,

Table 1.2 Production of hydrolytic extracellular enzymes from fungal endophytes

Fungal endophyte	Enzyme	Plant host	References
Colletrotrichum, *Fusarium*, *Phoma*, *Penicillium*	Asparaginase	*Cymbopogon citratus*, *Murraya koenigii*	Chow and Ting (2015)
Aspergillus	Amylase		Khan et al. (2017)
Pochonia chlamydosporia	Protease		Escudero et al. (2016)
Colletotrichum gloeosporioides	Amylase, chitinase, protease	*Camellia sinensis*	Rabha et al. (2014)
Acremonium, *Alternaria*, *Aspergillus*, *Chaetomium*, *Cladosporium*, *Colletotrichum*, *Cylindrocephalum*, *Discosia*, *Drechslera*, *Fusarium*, *Fusicoccum*, *Mycelia sterilia*, *Myrothecium*, *Nigrospora sphaerica*, *Paecilomyces*, *Pestalotiopsis*, *Phoma*, *Phyllosticta*, *Talaromyces emersonii*, *Xylaria*	Amylase, cellulase, laccase, lipase, pectinase, protease	*Alpinia calcarata*, *Bixa orellana*, *Calophyllum inophyllum*, *Catharanthus roseus*	Sunitha et al. (2013)
Aspergillus, *Bisporus*, *Chaetomium*, *Cladosporium*, *Colletotrichum*, *Curvularia*, *Fusarium*, *Rhizoctonia*	Amylase, cellulose, lipase, protease	*Azadirachta indica*, *Citrus limon*, *Gossypium*, *Magnolia*	Patil et al. (2015)
Cladosporium cladosporioides, *Colletotrichum carssipes*, *C. falcatum*, *C. gloeosporioides*, *Curvularia brachyspira*, *Drechslera hawaiiensis*, *Lasiodiplodia theobromae*, *Nigrospora sphaerica*, *Phyllosticta*, *Xylariales*	Amylase, cellulase, laccase, lipase, protease	*Adhatoda vasica*, *Coleus aromaticus*, *Costus igneus*, *Lawsonia inermis*	Amirita et al. (2012)
Amanita muscaria, *Boletus luridus*, *Hydnum rufescens*, *Lactariusa cerrimus*, *Piceirhiza bicolorata*, *Piloderma byssinum*, *P. fallax*, *Russulachloroides*, *Suillusluteus luteus*	Protease	Sporocarp	Nygren et al. (2007)
Colletotrichum sp., *Fusarium solani*, *Macrophomina phaseolina*, *Nigrospora sphaerica*	Amylase, cellulase, protease	*Catharanthus roseus*	Ayob and Simarani (2016)
Acremonium curvulum, *Aspergillus niger*, *Cochliobolus lunatus*, *Gibberella baccata*, *Myrmecridium schulzeri*, *Myrothecium verrucaria*, *Penicillium commune*, *Phoma putaminum*, *Pithomyces atro-olivaceus*, *Trichoderma piluliferum*	Cellulase, lipase, protease, xylanase	*Bauhinia forficata*	Bezerra et al. (2015)
Alternaria alternate, *Penicillium chrysogenum*	Amylase, cellulase	*Asclepias sinaica*	Fouda et al. (2015)

(continued)

Table 1.2 (continued)

Fungal endophyte	Enzyme	Plant host	References
Aspergillus terreus	L-aspar-aginase	*Sueada monoica*	Kalyanasundaram et al. (2015)
Hebelomaincarnatulum, *Laccaria bicolor*, *Phialocephala fortinii*, *Umbelopsis isabellina*	Protease		Mayerhofer et al. (2015)
Hormonema sp., *Neofusicoccum luteum*, *Neofusicoccum australe*, *Ulocladium* sp.	Laccase	*Eucalyptus*	Fillat et al. (2016)
Acremonium sp., *Alternaria* sp., *Aspergillus* sp., *Fusarium* sp., *Pestalotiopsis* sp.	Amylase, cellulase, lipase	*Acanthus ilicifolius*, *Acrostichum aureum*	Maria et al. (2005)

Cladosporium cladosporioides, *Fusarium lateritium*, *Nigrospora sphaerica, Penicillium aurantiogriseum, P. glandicola, Pestalotiopsis guepini*, and *Xylaria* sp.

Cabezas et al. (2012) isolated 100 fungal endophytes from *Espeletia* sp. and estimated their cellulolytic potential. The research showed that only four isolates could synthesize cellulases, of which *Penicillium glabrum* displayed the highest cellulolytic activity, with the highest CMCase, exoglucanase, and β-glucosidase enzyme activities of 44.5 U/ml, 48.3 U/ml, and 0.45 U/ml respectively. Syed et al. (2013) identified the endophytic fungus *Penicillium* sp. CPF2 (NFCCI 2862). Different substrates were assessed for optimal synthesis of cellulase by CPF2. The best activities for FPase (1.2 IU/ml), endocellulase (19 IU/ml), xylanase (40 IU/ml), and β-glucosidase (2.8 IU/ml) with a protein content of 0.86 mg/ml were detected when cellulose (1.5 % w/v) was used in association with peptone (0.2 % w/v) in the growth medium. Optimal temperature and pH for the extracellular cellulase production were 28 °C and 5.5 °C respectively. Onofre et al. (2013) evaluated the production of cellulases by endophytic fungi, *Fusarium oxysporum* isolated from *Baccharis dracunculifolia*. The results showed that after 55 days of fermentation, the maximum peak of enzyme production with a yield of 55.21 ± 10.54 IU/g of fermented substrate was at pH 5.96.

Patil et al. (2015) screened *Aspergillus* sp., *Bisporus* sp., *Chaetomium* sp., *Cladosporium* sp., *Colletotrichum* sp., *Curvularia* sp., *Fusarium* sp., and *Rhizoctonia* sp., isolated from seven medicinal plants and screened both qualitatively and quantitatively for the synthesis of hydrolytic extracellular enzymes, such as amylases, cellulases, lipases, and proteases. The study revealed that *Aspergillus* sp., *Bisporus* sp., *Cladosporium* sp., and *Colletotrichum* sp. showed cellulase production qualitatively, whereas quantitatively, *Rhizoctonia* sp. produced maximum cellulase of about 0.3 U/ml. However, other isolates, including *Bisporus* sp., *Chaetomium* sp., and *Fusarium* sp., exhibited moderate to low activity. Toghueo et al. (2017) reported the fungal endophytes from Cameroonian medicinal plants and screened for their extracellular cellulase activity. The two assays, enzyme and plate-clearing, were used for the screening of effective cellulolytic fungal endophytes. *Penicillium* sp., and *P. chermesimum* were the most effective producers.

1.3.1.2 Xylanase

Xylanases are glycosidases comprising endo-1,4-b-xylanaseand β-xylosidase and catalyzing the endohydrolysis of 1,4-b- D-xylosidic linkages in xylan (Collins et al. 2005; Thomas et al. 2017). These enzymes basically cause the hydrolysis of the xylan present in the hemicelluloses of plants and convert them into monomeric sugars; this function is not performed alone, but rather with the assistance of certain other hydrolytic enzymes, for instance, acetyl xylan esterase, α-L-arabinofuranosidase, α-glucuronidase, and phenolic acid, including ferulic and p-coumaric acid esterase (Collins et al. 2005; Thomas et al. 2017). The chief substrate of xylanases is xylan, which is the key structural polysaccharide of plant cells and the second most abundant polysaccharide in nature, accounting for approximately one third of all renewable organic carbon on earth (Collins et al. 2005; Prade 1996). Xylanases possess numerous applications in the food, de-inking, biofuels, baking, animal feed, and paper and pulp industries (Kumar et al. 2017a; Polizeli et al. 2005; Singh et al. 2016; Suman et al. 2015; Thomas et al. 2017). In the baking industry, xylanases improve the strength of the gluten and ultimately the superiority of the bread as they are capable of absorbing water and collaborating with gluten (Butt et al. 2008; Gray and Bemiller 2003; Harris and Ramalingam 2010; Nuyens et al. 2001). Xylanases are also used with other enzymes to improve the yield of juices from fruit and vegetables; the firmness of fruit pulp; and the regaining of aromas, edible dyes, essential oils, hydrolysis substances, mineral salts, etc. (Polizeli et al. 2005). These enzymes have been repoprted from different microorganisms such as algae, arthropods, bacteria, fungi, gastropods, and protozoa (Collins et al. 2005).

Wipusaree et al. (2011) isolated 54 endophytic fungi from the Thai medicinal plant, *Croton oblongifolius* Roxb, and screened the isolates for xylanase production. In primary screening, xylanase activity was found in 30 isolates by growing them on solid xylan agar plates. After secondary screening for xylanase activity in xylan liquid culture, the isolate with the highest xylanase production, identified as *Alternaria alternata*, was selected for further evaluation. The study revealed this xylanase to be monomeric, possessing molecular weight of 54.8 kDa. It showed a broadly similar substrate affinity to other xylanases, with a *Km* of 2.37 mg/ml, and was thermostable up to 40 °C. The enzyme was also shown to be inhibited to some extent by all tested divalent metal cations, but especially by Hg^{2+} and Cu^{2+}. Sorgatto et al. (2012) characterized xylanase synthesized by the endophytic fungus *Aspergillus terreus*, isolated from *Memora peregrine*. The research revealed an optimal temperature of 55 °C and a pH value of 4.5. The enzyme was thermotolerant at 45 °C and 50 °C, with a half-life of 55 and 36 min respectively. Tasia and Melliawati (2017) found *Acremonium* sp. and a member of the class *Coelomycetes* to be xylanase producers. The study by Marques et al. (2018) also reported *Acremonium* sp., *Botryosphaeria* sp., *Chaetomium* sp., *Cladosporium cladosporioides*, *Colletotrichum crassipes*, *Coniella petrakii*, *Coniothyrium minitans*, *Myrothecium gramineum*, *Paecilomyces* sp., *Phomopsis stipata*, *Saccharicola* sp., *Trichoderma viridae*, and *Ustilaginoidea* sp. to be xylanase producers.

1.3.1.3 Lipase

Lipases belong to serine hydrolases and do not require any cofactors. They are involved in diverse conversion reactions, such as transesterification, inter esterification, esterification, aminolysis, alcoholysis, and acidolysis (Gopinath et al. 2013; Panjiar et al. 2017; Savitha et al. 2007; Yadav et al. 2017a). Triacylglycerol acyl hydrolases are lipases that are involved in the hydrolysis of fats and oils (Gopinath et al. 2013; Singh and Mukhopadhyay 2012). Lipases are of great importance to the food industry. Phospholipases are being used in treating egg yolk, which is useful for the processing of baby foods, custard, dressings, and mayonnaise; for dough preparation; and for sauces, such as Hollandaise, Béarnaise, and Café de Paris (Aravindan et al. 2007; Reimerdes et al. 2004). Lipase-modified butter fat has extensive applications in different food processes (Aravindan et al. 2007; Uhlig 1998). Chocolates with cocoa butter substitutes, bread, structured lipids such as human milk fat replacers, low calorie health oils, and nutraceuticals are some of lipase-mediated food products available (Aravindan et al. 2007). The addition of lipases to noodles results in appreciably softer textural characteristics (Undurraga et al. 2001). Furthermore, lipases are also used to increase the flavor content of bakery products (Ray 2012).

Lipases are produced by bacteria, yeasts, protozoans, molds, and even viruses are known to encode genes for lipases (Abrunhosa et al. 2013; Anbu et al. 2011; Ginalska et al. 2004). The production of lipases has been demonstrated in ascomycetes and coelomycetes (Gopinath et al. 2013). Lipolytic activity has been shown in *Rhizopus* sp., *Penicillium* sp., *Mucor* sp., *Lipomyces starkeyi*, *Humicola lanuginose*, *Cunninghamella verticillata*, *Candida rugosa*, *Acremonium strictum*, and *Aspergillus* sp. (Tsujisaka et al. 1973; Jacobsen et al. 1990; Petrović et al. 1990; Sztajer and Maliszewska 1989). Microbial lipases are of commercial importance because of the broader availability, greater stability, and low production costs compared with plant and animal lipases.

Torres et al. (2003) rendered a mycelium-bound lipase from *Rhizopus oryzae* that catalyzed the esterification of fatty acids in iso-octane. The enzyme was active over the entire pH range studied, from pH 3 to pH 8, but maximal activity was obtained at pH 4 and pH 7. The study by Costa-Silva et al. (2011) deals with improvement in the production and stabilization of lipases from the endophytic fungi *Cercospora kikuchii* isolated from *Tithonia diversifolia*. Amirita et al. (2012) reported *Colletotrichum falcatum*, *Curvularia brachyspora*, *Curvularia vermiformis*, *Drechslera hawaiiensis*, and *Phyllosticta* sp. to be producers of lipase enzymes from different medicinal plants. Panuthai et al. (2012) screened 65 endophytic fungal isolates for the production of lipases, of which only 10 were found to produce extracellular lipases, with *Fusarium oxysporum*, isolated from the leaves of *Croton oblongifolius* Roxb. (Plao yai), yielding the highest level. The enriched lipase showed optimal activity at 30 °C and pH 8, and was reasonably stable up to 40 °C and at a pH of 8.0–12. Venkatesagowda et al. (2012) isolated species of *Trichoderma*,

Stachybotrys, *Sclerotinia*, *Rhizopus*, *Phyllosticta*, *Phomopsis*, *Phoma*, *Pestalotiopsis*, *Penicillium*, *Mucor*, *Lasiodiplodia*, *Fusarium*, *Drechslera*, *Curvularia*, *Colletotrichum*, *Cladosporium*, *Chalaropsis*, *Aspergillus*, and *Alternaria*, showing strong lipolytic activity. Sunitha et al. (2013) isolated lipase-producing *Acremonium implicatum*, *Alternaria* sp., *Aspergillus niger*, *Chaetomium* sp., *Colletotrichum falcatum*, *C. gleosporoides*, *C. truncatum*, *Cylindrocephalum* sp., *Drechslera* sp., *Fusarium oxysporum*, *Isaria* sp., *Mycelia streilia* sp., *Penicillium* sp., *Pestalotiopsis* sp., *Phoma* sp., *Phomopsis longicolla*, and *Xylaria* sp. from *Alpinia calcarata*, *Bixa orellana*, *Calophyllum inophyllum*, and *Catharanthus roseus.* Fareed et al. (2017) revealed *Aspergillus calidoustus*, *A. fumigates*, *Microsporum gypseum*, *Penicillium marneffei*, *P. viridicatum*, and *Trichophyton tonsurans* to be lipase producers.

1.3.1.4 β-glucosidase

Periconia sp. produce a thermotolerant β-glucosidase. This enzyme shows high activity toward cellobiose and carboxymethylcellulose. β-glucosidase hydrolyzes rice straw into simple sugars. Hydrolytic enzymes have the potential to convert lignocellulosic biomass to biofuels and chemicals (Harnpicharnchai et al. 2009). The major decomposers of lignocelluloses are fungi, which play an essential role in the cycling of carbon and other nutrients. Exo- and endoglucanases, exo- and endoxylanases, β-xylosidases, and β-glycosidase are the main hydrolytic enzymes involved in the degradation of lignocelluloses (Van Dyk and Pletschke 2012).

1.3.1.5 Tannases

Tannases comprise two classes of enzymes, tannin acyl hydrolases and ellagitannin acyl hydrolases, also called ellagitannases. Tannin acyl hydrolases are used in the beverage, food, leather, and pharmaceutical industries (González et al. 2017). Vegetable and animal tissues are easily available sources of tannases; however, on an industrial scale, microbial sources are preferred. Tannases have been obtained from fungi, including *Aspergillus* sp., *Paecilomyces variotii*, and *Penicillium* sp. (Battestin and Macedo 2007; González et al. 2017). There are some reports of tannase production by endophytic fungi. Cavalcanti et al. (2017) isolated 16 endophytic fungal strains and screened them for the production of tannases. All the isolates produced tannases, with *Aspergillus fumigatus* and *A. niger* being the highest producers. The study revealed that the optimal temperature and pH of enzymes from the two strains were 30 °C and 4.0 respectively.

1.3.1.6 Pectinases

Pectinase is an enzyme that actually breaks down pectin, which is a polysaccharide found in plant cell walls. This enzyme has shown a robust rise on the market and has also held a leading position amongst commercially produced industrial enzymes (Garg et al. 2016). In the industrial sector, this enzyme plays an important role in decreasing viscosity and improving yield (Garg et al. 2016; Makky and Yusoff 2015). In the processing of citrus juice, the enzyme helps to eliminate the cloudiness of the juice and stabilize it (Braddock 1981; Garg et al. 2016).

In wine processing, pectinases are used to promote filtration, increase the juice yield, and strengthen the flavor and color (Chaudhri and Suneetha 2012; Garg et al. 2016). Additionally, in biorefineries, pectinases used to hydrolyze pectin are present in agro-industrial waste (Biz et al. 2014; Garg et al. 2016). The agro-waste is converted into simple sugars and bioethanol, or could also be used as fermentable sugars (Alshammari et al. 2011; Garg et al. 2016). The fermentation of tea can be speeded up by breaking down the pectin present in the cell walls of tea leaves (Garg et al. 2016). Further, pectinases are used in textile processing, the extraction of vegetable oil, the processing of animal feed, the biobleaching of kraft pulp, and the recycling of wastepaper (Garg et al. 2016). The most important sources of pectinases include bacteria, fungi, and plants, and recently microbial pectinases have been gaining a lot of attention.

Sunitha et al. (2013) reported *Acremonium implicatum*, *Aspergillus fumigatus*, *Colletotrichum gleosporoides*, *Coniothyrium* sp., *Cylindrocephalum* sp., *Drechslera* sp., *Fusarium chlamydosporum*, *F. oxysporum, Fusicoccum* sp., *Nigrospora sphaerica*, *Paecilomyces variotii*, *Pestalotiopsis disseminata*, *Phoma* sp., *Pyllosticta* sp., *Talaromyces emersonii*, and *Xylaria* sp. to be pectinase producers isolated from *Alpinia calcarata*, *Bixa orellana*, *Calophyllum inophyllum*, and *Catharanthus roseus*. Fouda et al. (2015) isolated pectinase producers, including *Alternaria alternata*, *Penicillium chrysogenum*, and the third fungal strain, described as sterile hyphae from the medicinal plant of *Asclepias sinaica*. Heidarizadeh et al. (2018) produced pectinases from *Piriformospora indica.* After 6 days, the maximum dry cell weight was 10.21 g/L, the growth yield was about 0.65 g/g, the specific growth rate 0.56 day^{-1}, and pectinase activity was found to be 10.47 U/mL on pectin-containing medium (P^{+}). In another case of pectin-free medium (P^{-}), all parameters were kept lower than for P^{+} medium. It was found in the study that the synthesis of pectinase on P^{+} was 2.7 times greater than on the P^{-} medium (Maheshwari 2011). About 5 and 50 °C are the ultimate pH and temperature required for polygalacturonase activity respectively (Kirti and Reddy 2013; Singh 2006). Indeed, this is the leading note of synthesis of pectinase by *Piriformospora indica*; the optimal pH of enzyme was additionally submitted and noted as a would-be contender for imminent use in the fruit juice industries (Bezerra et al. 2012; Mercado-Blanco et al. 2016). Uzma et al. (2016) reported *Aspergillus* sp., *Cladosporium* sp., *Colletotrichum*

sp., *Fusarium* sp., *Mucor* sp., *Mycelia sterilia*, *Penicillium* sp., *Phoma* sp., *Phomopsis* sp., and *Rhizopus* sp. and found that these fungal species exhibited pectinase production attributes (Kaul et al. 2013; Kirti and Reddy 2013).

1.3.1.7 Phytases

Phytases, or myoinositol hexakisphosphate phosphohydrolase, are phytate-degrading enzymes. Phytases catalyze the hydrolysis of phytic acid to inositol phosphates, myoinositol, and inorganic phosphate (Gontia-Mishra and Tiwari 2013; Kaur et al. 2017; Kumar et al. 2016, 2017b; Mitchell et al. 1997). Phytases have been gaining a lot of interest and have become a center of focus for scientists and entrepreneurs in the fields of nutrition, environmental protection, and biotechnology (Yadav 2018; Yadav et al. 2017b, d). In plants, these enzymes are usually expressed during seed germination, bring about the degradation of the phytate, provide the growing seedling with orthophosphate, and lower inositol polyphosphates, free myoinositol, and previously bound cations, including Ca^{2+}, K^{+}, Mg^{2+}, and Zn^{2+}, and hence provide nutrition for plant growth (Gontia-Mishra and Tiwari 2013; Reddy et al. 1989). In animals, phytases play a role in the maintenance of the cell's metabolic reservoirs of inositol hexaphosphate and other inositol polyphosphates. Phytases have many applications. The activity of some yeasts and fungi is generally regarded as safe for consumption by humans and animals, for example, *Saccharomyces cerevisiae* (Gontia-Mishra and Tiwari 2013; Nayini 1984) could be used as a probiotic in a range of food formulations to improve the utilization of phosphate. Phytases can also be utilized in bakery products, especially in the bread-making process (Gontia-Mishra and Tiwari 2013; Haros et al. 2001). The addition of phytase is known to reduce the phytate content in dough and shorten the fermentation time. Further, it improves the bread shape, volume, and softness of the crumb. More phytases are also added in the fractionation of cereal bran, the absorption of iron, and in animal nutrition. In fact, numerous microbial phytases are already on the market and expansively used as animal feed supplements, for instance, phytase from *Aspergillus ficuum* as Natuphos, *A. niger* as Allzyme, *A. awamori* as Finase and Avizyme, *A. oryzae* as AMAFERM, SP, SF, TP, and Phyzyme, and *Peniophora lycii* as Ronozyme, Roxazyme, and Bio-Feed phytase (Gontia-Mishra and Tiwari 2013). Additionally, phytases are utilized in feed for fish, poultry, and pigs, as biofertilizers, in paper manufacturing, and in the wet milling of maize (Gontia-Mishra and Tiwari 2013). Although phytases have been described in plants, animals, and in a range of bacteria, filamentous fungi, and yeasts, here we concentrate primarily on those from endophytic fungi (Venugopalan and Srivastava 2015).

Marlida et al. (2010) obtained 34 isolates of fungal endophytes and screened them for phytase synthesis. Renuka and Ramanujam (2016) reported that phytase production could be achieved only in *Fusarium verticillioides* and *Rhizoctonia* sp., which were also best induced by phytic acid and rice bran compared with other inducers in the submerged fermentation medium used. The phytases produced by *Fusarium verticillioides* and *Rhizoctonia* sp. showed optimal pH of 5.0 and 4.0 respectively. Phytase from *F. verticillioides* showed an optimal temperature of 50 °C

and stability up to 60 °C, optimal pH at 5.0 and pH stability at 2.5–6.0. Mehdipour-Moghaddam et al. (2010) isolated *Azospirillum* strains from rice and wheat and screened the strains for cellulase, pectinase, and phytase activity. The study revealed that the *Azospirillum* strain isolated from rice showed considerably greater phytase activity than that isolated from wheat. In fact, to our knowledge, this is the first study to report phytase activity and its zymogram for *Azospirillum* with different activity profiles exhibited by various isolates.

1.3.1.8 Ligninolytic Enzymes

White rot fungi are the most efficient ligninolytic organisms described to date. Owing to the extracellular nonspecific and nonstereoselective enzyme system in white rot fungi, the ability to degrade lignin is more efficient (Barr and Aust 1994). Recently, some microorganisms isolated from the hardwood forests of Zimbabwe (Tekere et al. 2001), Tunisia (Dhouib et al. 2005), Spain (Barrasa et al. 2009), Northern China (Sun et al. 2011a), and Norway (Kim et al. 2015) have been reported in the production of ligninolytic enzymes. For the study of lignin-degrading enzymes in endophytes, different substrates such as ABTS (2,2'-azinobis-3-ethylbenzothiazoline-6-sulfonic acid), naphthol, and Poly R-478 have been isolated from living plants (Fillat et al. 2016; Oses et al. 2006; Sun et al. 2011a). From tree species *Drimys winteri* and *Prumnopitys andina*, endophytic fungi producing lignocellulolytic enzymes have been isolated. In *D. winteri*, *Bjerkandera* sp. and *Mycelia sterilia* (Dw-2) were identified, whereas in *P. andina*, an unidentified basidiomycete (Pa-1) and also *M. sterilia* (Pa-2) were recognized (Oses et al. 2006). Rodriguez et al. (2009) reported in the forest region that a basidiomycete and a deuteromycete use a combination of enzymes, 1,4-b-D-glucan cellobiohydrolases, endo-1,4-b-D-glucanases, and 1,4-b-D-glucosidase, which break glycoside linkages between B-D-xylopyranosyl and glucopyranosyl residues, thus promoting the biodegradation of wood.

The endophytic community of *Acer truncatum*, the main woody tree species of northern Chinese forests, was investigated, with 17 isolates belonging to the taxa *Alternaria alternata*, *A. arborescens*, *Ascochytopsis vignae*, *Coniothyrium olivaceum*, *Diaporthe* sp. 2, *Drechslera biseptata*, *Glomerella miyabeana*, *Gnomoniella* sp. 1, *Leptosphaeria* sp. 1, *Melanconis* sp. 1, *Melanconis* sp. 2, *Microsphaeropsis arundinis*, *Paraconiothyrium brasiliense*, *Phoma* sp. 4, *P. glomerata*, *Sirococcus clavigignenti-juglandacearum*, and *Coelomycetes* sp. reported to oxidize the substrate naphthol (Sun et al. 2011a). The medicinal plants *Adhatoda vasica*, *Costus igneus*, *Coleus aromaticus*, and *Lawsonia inermis* were collected from Sathyamangalam, Tamil Nadu (India) for the isolation of endophytic fungi and screened for the synthesis of laccase enzyme (Kaul et al. 2013; Vasundhara et al. 2016; Venugopalan and Srivastava 2015). The fungal isolates were grown on GYP agar medium amended with 1-naphthol. Out of 12 different species, only two endophytes, *Xylaria* sp. and *Curvularia brachyspora*, were positive in naphthol (Amirita et al. 2012). From the medicinal plants *Alpinia calcarata* Roscoe, *Calophyllum inophyllum* L, *Bixa orellana* L, and *Catharanthus roseus*, 50 strains of fungal

endophyte were isolated. Very few endophytic strains, *Phomopsis longicolla* (Bo13), *Discosia* sp. (Ci5), *Fusicoccum* sp. (Ac26), and *Chaetomium* sp., were able to produce laccase, i.e., showed oxidation of naphthol (Sunitha et al. 2013).

A total of 127 strains of fungal endophytes were isolated from *Eucalyptus globulus* trees of Spain: Cantabria, Asturias 128 (AS), Seville, (SE), Extremadura (EX), and Toledo (TO). Out of 127 strains of endophytic fungi, 21 showed positive ABTS oxidation in an agar plate medium containing ABTS. *Hormonema* sp., *Pringsheimia smilacis*, *Ulocladium* sp., *Neofusicoccum luteum*, and *N. australe* in liquid medium confirmed laccase production. Copper sulfate and ethanol were examined as inducers for increasing the production of laccase. *Pringsheimia smilacis* belonging to the family Dothioraceae were reported for the first time for the production of laccase (Bezerra et al. 2012; Fillat et al. 2016). *Trametes* sp. I-62 was optimized for the production of laccase and was applied to solve problems associated with pulp bleaching. Maximal laccase activity was obtained on the addition of wheat straw and copper sulfate in combination as inducers (Martin-Sampedro et al. 2013). Ligninolytic fungi were collected in Huejutla and characterized as having laccase activity as part of their fundamental enzymatic pool to mineralize lignin. Out of the 100 fungal isolates, 60 had laccase activity, indicating that the isolated fungi have great biotechnological potential (Ramírez et al. 2012).

Two isolates of *Fusarium proliferatum* from different global locations and ecological sites were reported to display similar abilities to degrade natural lignin from wheat (14C-MWL) and synthetic polymers (Anderson et al. 2005). Shi et al. (2004) demonstrated that the fungal endophyte *Phomopsis* sp. almost decays straw by degrading lignin. In another study, laccase and peroxide synthesized by fungal endophytes contributed reliably to the decomposition of litter lignin (Dai et al. 2010; Krishnamurthy and Naik 2017). Various researchers have observed the laccase activity of fungal endophytes in liquid medium: *Phomopsis liquidambari* (Diaporthaceae), *Xylaria* sp. (Xylariaceae), *Fusarium* sp., *F. proliferatum* (Nectriaceae), *Chaetomium* sp., *C. globosum* (Chaetomiaceae), *Podospora anserina* (Lasiosphaeriaceae), *Colletotrichum gloeosporioides* (Glomerellaceae), *Trichoderma harzianum* (Hypocreaceae), *Botryosphaeria* sp., *Neofusicoccum australe*, *N. luteum*, *Botryosphaeria rhodina* (Lasiodiplodia theobromae), *Botryosphaeria obtuse*, *B. dothidea*, *B. ribis* (Botryosphaeriaceae), *Monotospora* sp. (Hysteriaceae), and *Hormonema* sp. (Dothioraceae) (Anderson et al. 2005; Durand et al. 2013; El-Zayat 2008; Fillat et al. 2016; Muthezhilan et al. 2014; Sara et al. 2016; Srivastava et al. 2013; Urairuj et al. 2003; Wang J et al. 2006a; Xie and Dai 2015).

1.3.2 Bioresources for Secondary Metabolites

It is evident that numerous important compounds in the pharmaceutical and agronomy industries are synthesized by endophytes (Arora and Ramawat 2017). Numerous vital medicines have been acquired from plants, for instance, camptothecin, quinine, Taxol, vincristine, and vinblastine (Ramawat et al. 2009), whereas

more than 8500 bioactive metabolites with fungi as a source are well-known (Arora and Ramawat 2017; Demain and Sanchez 2009; Goyal et al. 2016). With the fungal endophyte *Taxomyces andreanae*, research into fungal endophytes was initiated, synthesizing certain bioactive molecules such as Taxol (Nicoletti and Fiorentino 2015). There are numerous tasks that have been encountered to synthesize these commercialized bioactive molecules (Arora and Ramawat 2017; Kusari and Spiteller 2011). In *Oryza sativa*, *Fusarium oxysporum* was reported to cause foolish seedling disease, as the fungus was also reported to produce gibberellin (Arora and Ramawat 2017). Further, for the synthesis of secondary metabolites, the bio-transformation process has been efficaciously realized using endophytes (Pimentel et al. 2011; Wang and Dai 2011). The process of chemical variation of any substance is referred to as bio-transformation in a biological system (Arora and Ramawat 2017; Wang and Dai 2011). The changes or transformation in the basic molecule result in a further effective active compound, e.g., semisynthetic compounds established from Taxol and podophyllotoxin have a supplementary effect to the basic molecule (Arora and Ramawat 2017; Ramawat et al. 2009). Figure 1.2 presents the chemical structures of secondary metabolites produced by different groups of endophytic fungi. Table 1.3 shows the diversity and abundance of diverse bioactive compound or secondary metabolite production by different groups of endophytic fungi reported from diverse host plants worldwide.

1.3.2.1 Azadirachtin

Azadirachtin is a recognized insecticide found in three species of the neem tree, *Azadirachta indica* A. Juss., *A. excelsa* Jacobs, and *A. siamensis* Valeton (Verma et al. 2014b). Azadirachtin is a highly oxygenated tetranortriterpenoid (Verma et al. 2014b). It contains 16 stereogenic centers, 7 of which are fully replaced (Ley et al. 1993). It takes about 16 years for its first structural interpretation and improvements (Butterworth et al. 1972) and 25 years for its chemical synthesis (Veitch et al. 2007a) to take place. Azadirachtin has been synthesized chemically from a common intermediate “epoxide-2”; this molecule alone has the potential as an intermediate to synthesize compounds from all three groups of limonoids: azadirachtin, azadirachtol, and meliacarpin from the neem tree (Kusari et al. 2012; Veitch et al. 2007b, c). Inside the cellular metabolism, azadirachtin is designed via the “iso-prenoid pathway” (IPP) (Kraus et al. 1985). Azadirachtin acts as an antifeedant, insect growth regulator, and sterilant in insects (Jennifer Mordue et al. 1998). Azadirachtin functions at a cellular level by disrupting protein synthesis, more precisely at the molecular level by altering the transcription and translation of protein expressed during rapid protein synthesis (Nisbet 2000). Azadirachtin has several structurally related isomers. Azadirachtin A and its several congeners have significant biological activity, specifically insecticidal and nematicidal (Klenk et al. 1986). To enable the synthesis of potential bioactive compounds, some novel biotechnological approaches have been used, such as callus culture (Krishnamurthy and Naik 2017; Prakash et al. 2002; Rafiq and Dahot 2010), cell culture (Jarvis et al. 1997), and hairy root culture of neem plants (Pimentel et al. 2011; Satdive et al. 2007).

Fig. 1.2 Structures of compounds presenting several novel bioactive secondary metabolites isolated from fungal endophytes

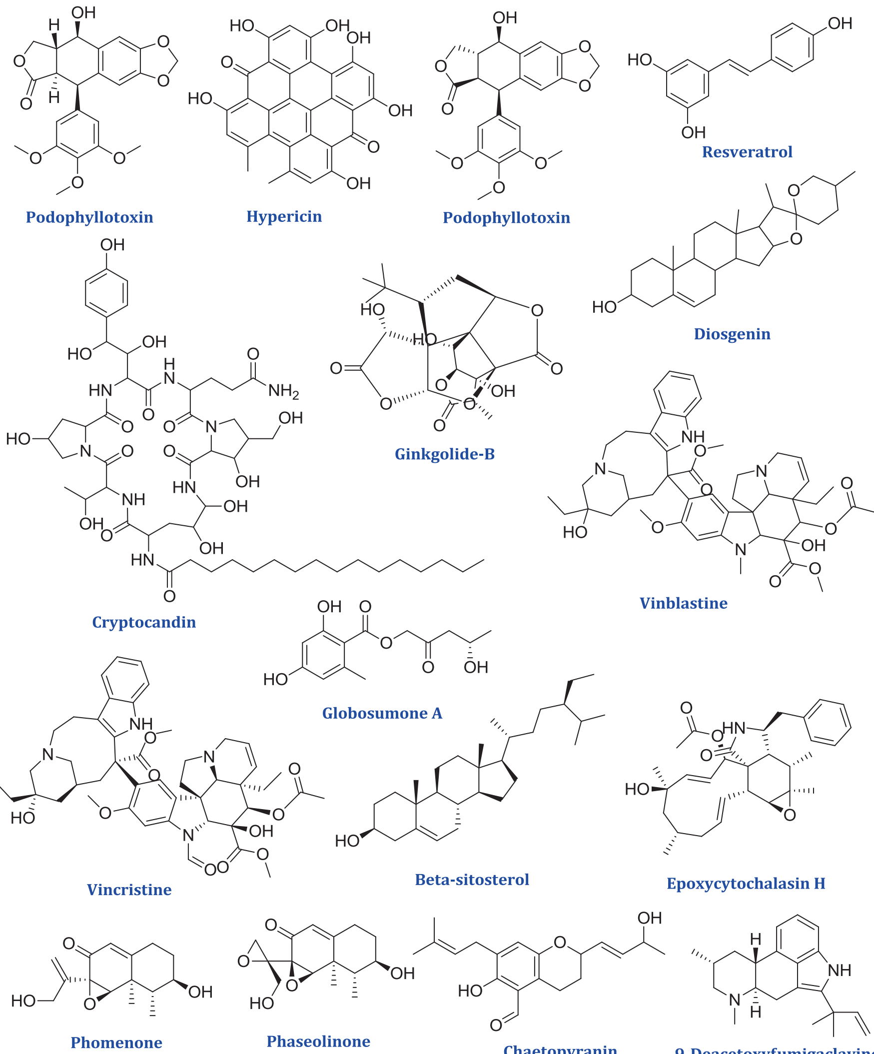

Fig. 1.2 (continued)

1.3.2.2 Camptothecin

Camptothecin (CPT) is a quinoline alkaloid mainly isolated from *Camptotheca acuminata*, a deciduous tree native to China and Tibet (Kumara et al. 2014). The bark of the tree was extensively used in traditional Chinese medicine (Wall et al. 1966). Later, camptothecin was discovered in several other species belonging to the families Icacinaceae, Rubiaceae, Apocynaceae, and Loganiaceae, with the maximum

Fig. 1.2 (continued)

concentration described in *Nothapodytes nimmoniana* (0.3% by dry weight from its bark) (Govindachari and Viswanathan 1972; Kumara et al. 2014). The biosynthetic pathway of CPT in plants is simply moderately categorized (Yamazaki et al. 2003, 2004). Further, Sun et al. (2011b) cloned and categorized three putative genes involved in CPT biosynthesis; namely, geraniol-10-hydroxylase, secologanin

Table 1.3 Production of bioactive compounds by endophytic fungi

Bioactive compounds	Host endophytic fungi	References
Antibacterial		
Altenuisol	*Alternaria* sp. Samif01	Tian et al. (2017)
Bacteriocins	*Bacillus subtilis*	Sansinenea and Ortiz (2011)
Chaetoglobosin A	*Chaetomium globosum*	Dissanayake et al. (2016)
Naphthaquinone	*Chloridium* sp.	Kharwar et al. (2009)
Polyketide citrinin	*Penicillium janthinellum*	Marinho et al. (2005)
Polyketide citrinin	*Penicillium janthinellum*	Marinho et al. (2005)
Phenols, flavonoids	*Pestalotiopsis neglecta*	Sharma et al. (2016)
Ambuic acid derivative	*Pestalotiopsis* sp.	Ding et al. (2008)
Phomodione (43)	*Phoma pinodella*	Hoffman et al. (2008)
Dicerandrol C (24)	*Phomopsis longicolla*	Erbert et al. (2012)
Solanioic acid	*Rhizoctonia solani*	Ratnaweera et al. (2015b)
Infectopyrones A and B	*Stemphylium* sp.	Zhou et al. (2014)
Ethanolic extract	*Trichoderma stromaticum*	Ratnaweera et al. (2015b)
Ophiobolin P	*Ulocladium* sp.	Wang et al. (2013)
Helvolic acid	*Xylaria* sp.	Ratnaweera et al. (2014)
Anticancer		
Vinblastine	*Alternaria*	Guo et al. (1998)
Capsaicin	*Alternaria alternata*	Clark and Lee (2016).
Resveratrol	*Aspergillus niger*	Liu et al. (2016)
Baccatin III	*Diaporthe phaseolorum,*	Li et al. (2015)
Secoemestrin D	*Emericella* sp.	Xu et al. (2013
Camptothecin	*Entrophospora infrequens*	Puri et al. (2005)
Asparaginase	*Eurotium* sp.	Jalgaonwala and Mahajan (2014)
Vincristine	*Fusarium oxysporum*	Kumar et al. (2013)
Vincristine	*Fusarium oxysporum*	Zhang et al. (2000)
Camptothecin	*Fusarium solani*	Shweta et al. (2010)
Camptothecin	*Fusarium solani*	Shweta et al. (2010)
10-hydroxycamptothecin	*Fusarium solani*	Shweta et al. (2010)
9-methoxycamptothecin	*Fusarium solani*	Shweta et al. (2010)
Torreyanic acid	*Pestalotiopsis microspora*	Lee et al. (1996)
Torreyanic acid	*Pestalotiopsis microspore*	Lee et al. (1996)
Podophyllotoxin	*Phialocephala fortinii*	Eyberger et al. (2006)
Podophyllotoxin	*Phialocephala fortinii*	Eyberger et al. (2006)
Taxol (paclitaxel)	*Taxomyces andreanae*	Kusari et al. (2014)
Antifungal		
Leucinostatin A	*Acremonium* sp.	Strobel et al. (1997)
Asperamide A, B	*Aspergillus niger*	Zhang et al. (2007)
Bacillomycin	*Bacillus amyloliquefaciens*	Aranda et al. (2005)
Bacilysocin	*Bacillus subtilis*	Tamehiro et al. (2002)
Gliotoxin	*Chaetomium globosum*	Li et al. (2011)
Cryptocandin	*Cryptosporiopsis quercina*	Strobel et al. (1999)

(continued)

Table 1.3 (continued)

Bioactive compounds	Host endophytic fungi	References
Cryptocin	*Cryptosporiopsis quercina*	Li and Strobel (2001)
Cryptocandin	*Cryptosporiopsis quercina*	Strobel et al. (1999)
Cytosporone B	*Dothiorella* sp.	Xu et al. (2005)
Enfumafungin	*Hormonema* sp.	Aly et al. (2011)
Microsphaerophthalide A	*Microsphaeropsis arundinis*	Sommart et al. (2012)
Sclerin	*Microsphaeropsis arundinis*	Sommart et al. (2012)
Ambuic acid	*Monochaetia* sp.	Li et al. (2001)
Myxodiol A	*Myxotrichum* sp.	Yuan et al. (2013)
Solanapyrone C	*Nigrospora* sp. YB-141	Wu et al. (2009)
Pinazaphilones A and B	*Penicillium* sp.	Liu et al. (2015)
Quinazoline alkaloid	*Penicillium vinaceum*	Zheng et al. (2012)
Jesterone	*Pestalotiopsis jester*	Li and Strobel (2001)
Pestaloside	*Pestalotiopsis microspore*	Lee et al. (1995)
Ambuic acid	*Pestalotiopsis* sp.	Li et al. (2001)
b-sitosterol	*Phoma* sp.	Wang et al. (2012)
Cytochalasin N	*Phomopsis* sp.	Fu et al. (2011)
Phomenone	*Xylaria* sp.	Silva et al. (2010)
Antimicrobial		
Altenusin	*Alternaria* sp.	Kjer et al. (2009)
Altersolanol A	*Ampelomyces* sp.	Aly et al. (2008)
Deoxypodophyllotoxin	*Aspergillus fumigatus*	Kusari et al. (2009)
Cephalosol	*Cephalosporium acremonium*	Zhang et al. (2008)
Javanicin	*Chloridium* sp.	Kharwar et al. (2009)
Methanol	*Colletotrichum* sp.	Arivudainambi et al. (2011)
Cytonic acid	*Cytonaema* sp.	Guo et al. (2000)
Cytonic acid B	*Cytonaema* sp.	Li et al. (2007b)
Eupenicinicols	*Eupenicillium* sp.	Li et al. (2014)
Equisetin	*Fusarium* sp.	Ratnaweera et al. (2015a)
Gliotoxin	*Hypocrea virens*	Ratnaweera et al. (2016)
Bisdethiobis gliotoxin	*Hypocrea virens*	Ratnaweera et al. (2016)
Botralin	*Microsphaeropsis olivacea*	Li et al. (2007b)
Graphislactone A	*Microsphaeropsis olivacea*	Li et al. (2007b)
Ulocladol	*Microsphaeropsis olivacea*	Li et al. (2007b)
Isocaryophyllene	*Muscodor sutura*	Kudalkar et al. (2012)
Octadecylmorpholine	*Muscodor tigerii*	Saxena et al. (2015b)
Phomol	*Phomopsis* sp	Weber et al. (2004)
Antioxidant		
Campothecin	*Entrophospora*	Puri et al. (2005)
3-*epi*-dihydroaltenuene	*Alternaria* sp.	Tian et al. (2017)
Pestacin and isopestacin	*Pestalotiopsis microspora*	Harper et al. (2003)
Isopestacin	*Pestalotiopsis microspora*	Strobel et al. (2002)
Pestacin	*Pestalotiopsis microspora*	Strobel et al. (2002)

(continued)

Table 1.3 (continued)

Bioactive compounds	Host endophytic fungi	References
Antitumor		
Naptha-y-pyrone	*Aspergillus niger*	Zhang and Qi-Yong (2007)
Cytochalasins	*Rhinocladiella* sp.	Wagenaar et al. (2000)
Cytochalasins	*Xylaria* sp.	Wagenaar et al. (2000)
Antiviral		
Podophyllotoxin	*Alternaria* sp.	Eyberger et al. (2006)
Cytonic acid A	*Cytonaema* sp.	Guo et al. (2000)
Pestalol A–E	*Pestalotiopsis* sp.	Sun et al. (2014)
Cytotoxic		
Cordyheptapeptides C–E	*Acremonium persicinium*	Chen et al. (2012)
Desmethyldiaportinol	*Ampelomyces* sp.	Aly et al. (2008)
8-O-methylversicolorin	*Aspergillus versicolor*	Dou et al. (2014)
Xanthoquinodin	*Chaetomium elatum*	Chen et al. (2013)
Coniothiepinol A	*Coniochaeta* sp.	Wang et al. (2010a)
Conioxepinol B	*Coniochaeta* sp.	Wang et al. (2010b)
Myxotrichin A	*Myxotrichum* sp.	Yuan et al. (2013)
Myxotrichin D	*Myxotrichum* sp.	Yuan et al. (2013)
Terricollene A	*Neurospora terricola*	Zhang et al. (2009a)
Ginsenocin	*Penicillium melinii*	Zheng et al. (2013)
Penicillenols A_1 and B_1	*Penicillium* sp.	Lin et al. (2008a)
Torreyanic acid	*Pestalotiopsis microspora*	Lee et al. (1996)
Phaeosphaerin A	*Phaeosphaeria* sp.	Li et al. (2012a)
Preussochrome C	*Preussia africana*	Zhang et al. (2012)
Atrichodermone A, B, C	*Trichoderma atroviride*	Zhou et al. (2017)
Immunosuppressive		
Subglutinol A and B	*Fusarium subglutinans*	Lee et al. (1995)
Periconicins	*Fusarium subglutinans*	Kim et al. (2004)
Insecticidal		
Azadirachtin A	*Eupenicillium parvum*	Kusari et al. (2012)
Azadirachtin	*Eupenicillium parvum*	Kusari et al. (2012)
Nodulisporic acid A	*Nodulisporium* sp	Ondeyka et al. (1997)
1,3-oxazinane	*Geotrichum* sp. AL4	Li et al. (2007a)
4-hydroxybenzoic acid	*Fusarium oxysporum*	Bogner et al. (2017)

synthase, and strictosidine synthase from *C. acuminata*. In recent times, an effort was made to unravel the CPT biosynthetic gene from a CPT-producing endophytic fungus, *Fusarium solani*, isolated from *C. acuminata* (Kusari et al. 2011; Kaul et al. 2013; Kumara et al. 2014). However, the endophyte was revealed to synthesize CPT. Kusari et al. (2011) suggested that the endophyte might be using the host STR to synthesize CPT. However, as Sudhakar et al. (2013) debated, this proposition is unbelievable, because the endophyte was able to produce CPT in axenic cultures for numerous generations in the absence of the host tissue, where evidently the fungus

cannot access the host *STR*. Anticancer drugs isolated from endophytic fungi include camptothecin, which is a potent anti-neoplastic agent isolated from *C. acuminata* Decaisne (Nyssaceae) from China (Premjanu and Jayanthy 2012; Wall et al. 1966).

1.3.2.3 Taxol

Paclitaxel, a greatly functionalized diterpenoid, occurs in *Taxus* plants (Suffness 1995). Derivatives of paclitaxel signify a leading group of anticancer agents that were earlier reported to be synthesized by endophytes (Kumara et al. 2014). In plants, the synthesis of Taxol occurs by the involvement of three genes, namely, *ts* (involved in the formation of the taxane skeleton), *dbat* (involved in baccatin-III formation), and *bapt* (involved in phenylpropanoyl side chain formation at C-13) (Xiong et al. 2013). Zhang P et al. (2009b) reported the gene 10-deacetylbaccatin-III-10-O-acetyl transferase to be accountable for the biosynthesis of Taxol in the endophyte *Cladosporium cladosporioides* MD213 isolated from *Taxus media* (yew species). In recent times, Xiong et al. (2013) revealed that in three Taxol-synthesizing endophytes isolated from *Anglojap* Yew, or *T. media*, the fungus resulted in positive successes for the three key genes, *ts*, *dbat*, and *bapt*. The fungus *Taxomyces andreanae*, an endophyte isolated from *T. brevifolia*, was found to produce Taxol (Stierle et al. 1993), subsequently drawing the attention of microbiologists to endophytes. Each plant is a repository of one or more fungal endophytes, and one endophytic species may possess several to a few hundred strains (Huang et al. 2007; Strobel and Daisy 2003). In recent years, the biosynthetic potential of endophytic fungi has gained more significance. It is thus imperative to study the complex relationship of endophytes with existing endophytes, host plants, insect pests, and other definitive herbivores, which standardizes the ability of endophytes to synthesize compounds similar to their hosts (Kusari et al. 2013b). *Aegle marmelos*, an extensively used medicinal plant, shelters Taxol-producing fungi (Gangadevi and Muthumary 2008). Taxol is an important and expensive anticancer drug generally used in clinics. The endophytic fungus *Bartalinia robillardoides* (strain AMB-9) produces 187.6 l g/l of Taxol. This confirms that the fungus can be genetically upgraded to increase the synthesis of Taxol. Taxanes such as Taxol are plentifully synthesized by members of the coniferous family Taxaceae (Wang and Dai 2011). It was found that a number of fungal endophytes isolated from yew trees (*Taxus* spp., Taxaceae) produce Taxol under in vitro conditions (Zhou et al. 2010).

1.3.2.4 Gibberellic Acid and Indole Acetic Acid

The biosynthetic pathway of gibberellic acid (GA) is compared with other secondary metabolites (Kumara et al. 2014). In plants, the conversion of GGDP to active GA requires the presence of three terpene synthases, two 450s, and a soluble 20 DDS.

Compared with the fungus, the synthesis is made by only one bifunctional terpene cyclase (copalyl synthase/kaurene synthase) and by P450s. These results suggest that the biosynthetic pathways in plants and fungi might have evolved independently (Bömke and Tudzynski 2009; Kumara et al. 2014). GA production has also been reported from the endophytic fungus *F. proliferatum*, isolated from orchid roots. Research has specified that this fungus obtained the genes for GA biosynthesis from higher plants by horizontal gene transfer. Endophytic microorganisms have been found to produce phytohormones such as GA, abscisic acid, auxins, cytokinins, and ethylene (Kaul et al. 2013).

Hamayun et al. (2009b) isolated *Cladosporium sphaerospermum* from the roots of *G. max* (L.), which indicated the presence of bioactive GA3, GA4, and GA7. The endophytes isolated from medicinal plants have been found to encourage plant growth and development. Waqas et al. (2012) studied the endophytic fungi *Phoma glomerata* and *Penicillium* sp. in the promotion of shoot growth, related vegetative growth, and other characteristics of GA-deficient dwarf mutant Waito-C and Dongjin-byeo rice. Therefore, if cultured endophytes were to produce the same rare and important bioactive compounds as their host plants, this would diminish the harvesting of slow-growing rare plants, and also help to restore the world's biodiversity (Waqas et al. 2012). Jerry (1994) revealed that during seed germination, the symbiotically associated endophytic fungi degrade cuticle cellulose and make carbon available to seedlings, which improves seed germination, vigor, and establishment. Endophytes have the ability to produce plant growth regulators and thereby promote seed germination in crop plants (Bhagobaty and Joshi 2009). Plant growth promotion is the major contribution of fungal symbiosis (Hassan et al. 2013). However, fungal endophytes enhance plant growth by the production of ammonia and plant hormones, particularly IAA (Bal et al. 2013). IAA acts as a plant growth promoter that enhances both cell elongation and cell division, and is essential for plant tissue differentiation (Taghavi et al. 2009). The ability of soil microorganisms to become involved in the production of IAA in culture plates and in soil has been recorded (Spaepen and Vanderleyden 2011). The endophytic microorganisms isolated from various plants have shown a high IAA production level compared with those isolated from root-free soil (Spaepen et al. 2007). Owing to the impact of IAA on the plant tissues, the ability of fungal endophytes to produce IAA has provoked a great response (Hamayun et al. 2010). Only a few fungi linked with plants have been stated to synthesize gibberellin (Kawaide 2006; Vandenbussche et al. 2007), for instance, *Cladosporium sphaerospermum* and *Penicillium citrinum* (Hamayun et al. 2009b; Khan et al. 2008). Hamayun et al. (2010) examined gibberellin production and the growth-promoting potential of a fungal strain belonging to *Cladosporium* sp. isolated from the roots of the cucumber. Khan et al. (2008) isolated *P. citrinum*, which showed growth promotion activity in dune plants owing to the presence of bioactive gibberellins in the filtrate of the fungi (Khan et al. 2008). Hasan (2002) revealed the growth promotion activity of endophytic *Phoma herbarum* and *Chrysosporium pseudomerdarium* in the soybean and proved that some endophytes are host-specific. Ahmad et al. (2010) studied the plant growth-promoting activity and stress resistance capability of endophytic *Penicillium* sp. and

Aspergillus sp., which were shown to produce physiologically active gibberellins. Many fungal endophytes, such as *Neurospora crassa* (Rademacher 1994), *Sesamum indicum* (Choi et al. 2005), *Penicillium citrinum* (Khan et al. 2008), *Scolecobasidium tshawytschae* (Hamayun et al. 2009b), *Arthrinium phaeospermum* (Khan et al. 2009a), *Chrysosporium pseudomerdarium* (Hamayun et al. 2009a), *Cladosporium sphaerospermum* (Hamayun et al. 2009c), *Cladosporium* sp. (Hamayun et al. 2009c), *Gliomastix murorum* (Khan et al. 2009b), *Fusarium fujikuroi*, *Sphaceloma manihoticola* (Shweta et al. 2010), *Phaeosphaeria* sp. (Kawaide 2006), *Phaeosphaeria* sp., *Penicillium* sp. (Hamayun et al. 2010), *Aspergillus fumigatus* (Khan et al. 2011a), *Exophiala* sp. (Khan et al. 2011b), and *P. funiculosum* (Khan et al. 2011b), have been reported to be gibberellin producers. Hasan (2002) demonstrated gibberellin production with molds such as *Aspergillus flavus*, *A. niger*, *Penicillium corylophilum*, *P. cyclopium*, *P. funiculosum*, and *Rhizopus stolonifera.*

1.3.2.5 Siderophores

Endophytes help plants to take up solubilized phosphate (Wakelin et al. 2004), enhancing hyphal growth and mycorrhizal colonization (Will and Sylvia 1990), and by producing siderophores (iron-chelating molecules that increase the availability of phosphate to plants) (Costa and Loper 1994). Endophytic bacteria were found to be responsible for the allelopathic effects on maize observed with these plants, causing reduced plant emergence and plant height (Sturz et al. 1997). Dutta et al. (2008) reported improvement of plant growth and disease suppression in pea plants co-inoculated with fluorescent pseudomonads and *Rhizobium*. Hung et al. (2007) studied the effect of endophytes on soybean growth and development, and proved them to have a positive influence on root weight. Plant growth-promoting endophytic bacteria influence seed germination, root and hypocotyl growth and increase seedling vigor. Root endophytes in the cortical parenchymatous tissue of vetiver were used for the enhancement of essential oil metabolism (Del Giudice et al. 2008). Harish et al. (2009) studied the effect of the bio-formulations of consortial combinations of the rhizobacteria *Pseudomonas fluorescens* (Pf1) and endophytic *Bacillus* sp. (EPB22), which enhanced the yield of bananas. One of the bacterial endophytes, *B. subtilis* HC8, isolated from hogweed, *Heracleum sosnowskyi*, found the potential to stimulate plant growth and the biological control of foot and root rot diseases in tomato (Malfanova et al. 2011).

In field experiments, inoculation with endophytic bacteria resulted in sugarcane plants that were more superior in terms of plant height and shoot counts. Conventional manipulation of soil microorganisms has been practiced for decades. For example, sewage and manure applications for the enhancement of soil fertility dramatically affect autochthonous communities of soil biota (Biswas et al. 2018). The practice of monoculture is in itself instrumental in altering soil microbial populations at the field level. Thus, it may be possible to influence plant endophytic populations by seed bacterization, by soil inoculation, and by identifying the genetic (bacterial) component responsible for their beneficial effects. Endophytic microbes have merit over rhizospheric bacteria, as they deliver fixed nitrogen straight to the host plant

tissue and are able to fix nitrogen more competently than free-living bacteria because of the lower oxygen pressure in the interior of plants than in soil. Jha et al. (2013) explored the potential of endophytic association with plants in agricultural sustainability in particular and yield enhancement in general. The potential of biofertilizers was formulated using endophytic bacteria for the enhanced production of bananas in a sustained way (Ngamau et al. 2014).

1.3.3 Pharmaceutically Important Bioactive Compounds

Throughout the year, natural products from microorganisms, plants, or animals play a key role in the search for novel drugs. These naturally derived products are non-toxic and inexpensive, and have been exploited for human use. The biggest store of bioactive compounds is fungal endophytes. Alexander Fleming, in 1928, discovered the first bioactive compound from *Penicillium notatum*, i.e., penicillin. During the 1990s one of the most useful anticancer drugs was paclitaxel. An endophyte of *T. brevifolia*, *Taxomyces andreanae* was reported to produce the drug paclitaxel. Later research suggested lateral gene transfer from host to fungus (Stierle et al. 1993). The fungal endophyte *Fusarium* was reported to produce subglutinol A and diterpene pyrones, providing immunosuppressive activity. The endophyte was isolated from the stem of *Tripterygium wilfordii* (Strobel and Pliam 1997). Isobenzofuranone as isopestacin, obtained from the fungal endophyte *Pestalotiopsis microspora*, possesses antifungal and antioxidant activity (Strobel et al. 2002). Antimicrobial activity of fungal endophytes was screened against the pathogenic organisms *Staphylococcus aureus*, *Candida albicans*, and *Cryptococcus neoformans.* Fungal endophytes were isolated from the leaves and branches of five different species of *Garcinia* plants. The fungal endophytes *Phomopsis* sp. and *Botryosphaeria* sp. showed antibacterial activity against *Staphylococcus aureus. Botryosphaeria* sp. also showed antifungal activity against *M. gypseum.* The results specify that the endophytic fungus of *Garcinia* plants are a potential source of antimicrobial compounds (Phongpaichit et al. 2006).

Endophytic fungi can be isolated from the bark of *Juglans mandshurica.* On the basis of the internal transcribed spacer sequence and morphological identification, the fungal endophyte belongs to Deuteromycotina, Hyphomycetes, Moniliales, and *Trichoderma longibrachiatum*. The fermentation of fungus FSN006 provides a possible mechanism for producing anticancer drugs with lower toxicity and greater efficiency (Li et al. 2009). The crude extract of the endophytic fungus *Pichia guilliermondii* was separated using bioassay-guided fractionation. Helvolic acid exhibited strong, broad-spectrum, antimicrobial activity (Zhao et al. 2010). Developments in screening technologies have received much attention; thus, fungal endophytes are an outstanding source of biologically active compounds with applications in medicine and agriculture (Aly et al. 2011). A large number of bioactive compounds produced by fungal endophytes are alkaloids, steroids, terpenoids, peptides, polyketones, flavonoids, quinols, phenols, xanthones, chinones, isocoumarins, benzopyranones, tetralones, cytochalasines, perylene derivatives, furandiones, depsipeptides,

and enniatins (Elfita et al. 2011) Tenguria et al. (2011) reported that endophytic fungi of *Tinospora crispa* (L.) was a probable candidate for the synthesis of bioactive compounds. *Plasmodium* species cause the most acute diseases in human beings, and even death. Hypericin isolated from fungal endophytes of medicinal plants possess antimicrobial activity against *Staphylococcus* sp., *Klebsiella pneumoniae*, *Pseudomonas aeroginosa*, *Salmonella enteric*, and *Escherichia coli* (Kusari and Spiteller 2012).

Khan et al. (2012a) reported five fungal endophytes isolated from *Capsicum annuum, Cucumis sativus*, and *G. max* roots. Using phylogenetic analysis, the isolate was found to belong to *Paraconiothyrium* sp. and produce the phytotoxic compound ascotoxin characterized using gas chromatography-mass spectrometry and the nuclear magnetic resonance technique. On seed germination of *Echinochloa crus-galli* and *Lactuca sativa*, 100% inhibitory effects were shown by ascotoxin. The buds and leaf of Malabar Embelia, found in peninsular India, were subjected to the isolation of fungal endophytes. Four different fungal endophytes were identified, *Cladosporium cladosporiodes*, *Penicillium* sp., *Aspergillus niger*, and *Alternaria* sp., and were characterized for phytochemical analysis and antibacterial activity against *Pseudomonas aeuroginosa*, *Bacillus subtilis*, and *Shigella flexneri.* The four different fungal endophytes exhibited the presence of phytochemicals at different concentrations: cardiac glycoside, flavonoids, phenols, tannins, terpenoids, cardenolides, and saponins. Endophytic microbes are a great source of bioactive compounds to satisfy the demands of the pharmaceutical and medical industries (Chandrappa et al. 2013). *Pinellia ternata* is used as a traditional medicine for antiemetic and sedative effects, and as an antitussive and analgesic. Su et al. (2014) isolated 193 endophytic microbes from Chinese medicinal plants, *Camptotheca cuminata* Decne, *Gastrodia elata* Blume, and *Pinellia ternata.* On the basis of morphological and rDNA sequences, the fungal isolates belong to Ascomycota, Basidiomycota, and Mucoromycotina. Endophytes produce various types of compounds, for instance, essential oils, azadirachtins, terpenes, flavonoids, lignins, cytochalasins, steroids, and alkaloids (Nicoletti and Fiorentino 2015).

1.3.4 Lignocellulosic Biorefineries: Biofuel Production

One of the main renewable materials on earth is wood. The cell walls of wood are composed of cellulose microfibrils covered with hemicelluloses and lignin hemicellulose matrices (Higuchi 2012). In 1813, Swiss botanist, A. P. de Candolle, mentioned lignin for the first time. About 20–30% of the dry weight of wood is made up of lignin (Abdel-Raheem and Shearer 2002). It is covalently linked to hemicellulose and confers mechanical strength to the cell wall (Chabannes et al. 2001). Owing to the chemical complexity and recalcitrant properties of lignin, very few microbes are able to degrade it (Guillén et al. 2005). In biorefinery processes, such as the production of ethanol and cellulose-based papers, the degradation of lignin is a central issue (Cañas and Camarero 2010).

An array of extracellular oxidative enzymes are produced by white-rot fungi (basidiomycetes), as they are the main wood rotters that synergistically and proficiently degrade lignin. Ligninolytic enzymes include lignin peroxidases (LiPs), manganese peroxidases (MnPs), versatile peroxidases, and laccases (Wong 2009). On the basis of macroscopic features, wood-rotting basidiomycetes are categorized into white-rot and brown-rot fungi (Schwarze et al. 2000). In the mid-1980s, LiP and MnP were discovered in *P. chrysosporium* and termed true ligninases because of their high redox potential (Evans et al. 1994). *Pleurotus eryngii* were reported to produce versatile peroxidase that showed catalytic properties similar to LiP and MnP (Ruiz-Dueñas et al. 1999). Other extracellular enzymes involved in wood lignin degradation are oxidases generating H_2O_2, aryl-alcohol oxidase (AAO), glyoxal oxidase, aryl-alcohol dehydrogenases (AAD), and quinone reductases (QR) (Guillén et al. 1997; Gutierrez et al. 1994).

Laccases have been known for many years to play a variety of roles, including production of pigments, fruit body morphogenesis, lignification of cell walls, and detoxification in plants, fungi, and insects (Mayer and Staples 2002). The preliminary steps in the biodegradation of lignin must be extracellular. LiP is also called a ligninase. First discovered in *Phanerochaete chrysosporium*, this enzyme is a heme peroxidase with a remarkably high redox potential and low optimal pH (Tien 1987). Laccase enzymes are copper-containing oxidases that mostly oxidize only those lignin model compounds with a free phenolic group, forming phenoxy radicals (Bourbonnais and Paice 1990). The most common laccase-producing endophytic fungi are *Chaetomium* sp., *C. globosum*, *Podospora anserina*, *Botryosphaeria* sp., and *Neofusicoccum austral* (Fillat et al. 2016; Sara et al. 2016).

Laccase enzymes produced by endophytic fungi have extensive substrate specificity and generally act on small organic substrates, such as polyphenols, methoxy-substituted phenols, and aromatic amines. Fungal laccases are used in paper manufacture for delignification, bioremediation of phenolic compounds, and biobleaching (Kunamneni et al. 2008). Exoglucanases, endoglucanases, β-glycosidase, exoxylanases and endoxylanases, and β-xylosidases are the main hydrolytic enzymes involved in lignocellulose degradation (Van Dyk and Pletschke 2012). For complete degradation of lignocellulose materials, laccases, MnP and LiP (oxidative enzymes), and additional hemicelluloses (e.g., acetyl esterase, b-glucuronidase, endo-1, 4-β-mannanase, and α-galactosidase) and oxidoreductases (aryl-alcohol oxidase, glucose-1-oxidase, glyoxal oxidase, and pyranose-2-oxidase) are necessary (Correa et al. 2014).

1.3.5 Endophytic Fungi in Bioremediation

Bioremediation is a process used to treat contaminated media, including water, soil, and subsurface material, by varying the conditions of the environment to stimulate the growth of microorganisms (fungi or bacteria) and degrade the target pollutants into simpler compounds. Biological treatment of the contaminated site is the least

expensive method (Barranco et al. 2012). To optimize the conditions for the microorganisms, additional nutrients, vitamins, minerals, and pH buffers are added. The prime goal of bioremediation is to create an optimal environment for the microbes to degrade pollutants. Although it is a cost-effective option, it is a very slow process, sometimes taking weeks to months for results to appear. Technologies can be generally classified as in situ or ex situ. In situ bioremediation involves treating the contaminated material at the site, whereas ex situ involves the removal of the contaminated material to be treated elsewhere.

To restrain the growth of endophytes, the plant synthesizes a range of toxic metabolites and endophytes over a period of co-evolution, progressively establishing a genetic system as a tolerant mechanism by generating exoenzymes and mycotoxins (Mucciarelli et al. 2007; Pinto et al. 2000). Fungal endophytes synthesizing the enzymes degrade the macromolecules into simpler compounds, including amylases, lipases, pectinase, cellulase, proteinase, phenol oxidase, and lignin catabolic enzymes (Oses et al. 2006; Tan and Zou 2001; Zikmundova et al. 2002). In general, fungal endophytes have been stated to have the ability to use various organic compounds, such as glucose, oligosaccharides, cellulose, hemicelluloses, lignin, keratin, pectin, lipids, and proteins, allowing the degradation of structural components into simpler forms (Kudanga and Mwenje 2005; Tomita 2003; Urairuj et al. 2003).

One of the methodologies in which green plants are used for the process of bioremediation is referred to as phytoremediation. It has been documented to be a promising technology for the in situ remediation of contaminated soils. Numerous studies have demonstrated that endophytes produce various enzymes for the degradation of organic contaminants and reduce both the phytotoxicity and evapotranspiration of volatile contaminants (Li et al. 2012b). Soleimani et al. (2010) reported the infection of *Festuca pratensis* and *Festuca arundinacea*, two grass species, by two endophytic fungi, *Neotyphodium coenophialum* and *Neotyphodium uncinatum*, increasing the ability of the plants to accumulate more Cd in roots and shoots and decreasing stress in the plants in addition to increasing the production of biomass. Rabie (2005) reported the phytoremediation efficiency of wheat, mung beans, and eggplant grown in soil contaminated with hydrocarbons. He concluded that the plants provided a larger sink for the contaminants, because they were better able to survive and grow, leading to the significance of treatment with arbuscular mycorrhizal fungi.

Since the industrial revolution, there has been a widespread rise in the discharge of waste into the environment, which is mostly collected in soil and water, comprises heavy metals, and generates distressing conditions for human life and aquatic biota. Heavy metals are metals with relatively high densities, atomic weights, or atomic numbers. Some heavy metals are either vital nutrients, such as iron, cobalt, and zinc, or comparatively harmless, such as ruthenium, silver, and indium, but in higher amounts or definite forms they can be toxic. Cadmium, mercury, and lead are reported to be highly poisonous heavy metals. Salem et al. (2000) reported that

arsenic, cadmium, chromium, copper, lead, mercury, nickel, selenium, silver, zinc, etc., are not only cytotoxic, but also carcinogenic and mutagenic (Ahluwalia and Goyal 2007). In heavy metal-polluted habitats, microorganisms are known to change different detoxifying mechanisms, such as biosorption, bioaccumulation, biotransformation, and biomineralization (Gadd 2000; Lim et al. 2003; Malik 2004).

One of the biological processes in which chemical changes on compounds take place is referred to as biotransformation. The endophytic fungus *Phomopsis* sp. (VA-35), obtained from *Viguiera arenaria*, was reported to biotransform the tetrahydrofuran lignan, (-)-grandisin, into a new compound, 3,4-dimethyl-2-(4'-hydroxy-3',5'-dimethoxyphenyl)-5-methoxy-tetrahydrofuran (Verza et al. 2009). In another study, endophytic fungi *Fusarium sambucinum*, *Plectosporium tabacinum*, *Gliocladium cibotii*, and *Chaetosphaeria* sp., isolated from the roots and shoots of *Aphelandra tetragona*, were capable of transforming the benzoxazinones 2-benzoxazolinone (BOA) and 2-hydroxy-1,4-benzoxazin-3-one (HBOA). Aminophenol was formed as a key intermediate during the metabolic pathway for HBOA and BOA degradation (Zikmundova et al. 2002). On the basis of 18S rRNA gene sequencing, *Lasiodiplodia theobromae* isolated from the leaves of *Boswellia ovalifoliolata*, an endemic medicinal plant from the Tirumala Hills, was reported to show resistance to all four heavy metals, Co, Cd, Cu, and Zn, up to 600 ppm (Sani et al. 2017).

1.3.6 Endophytic Fungi in Agriculture

A lot of research into fungal endophytes is underway, which signifies that they are the most important source of biocontrol agents. They have a considerable effect on the physiological actions of their host plants. Further, various environmental factors, including rainfall and humidity, may have an influence on the occurrence of some fungal endophytic species (Khiralla et al. 2017; Petrini 1991; Selvanathan et al. 2011). According to Schaechter (2012), endophytic fungi have frequently been categorized into two major groups, including clavicipitaceous endophytes, which are known to infect some grasses, and nonclavicipitaceous endophytes. The Clavicipitaceae family of fungi include free-living and symbiotic species in association with insects and fungi or grasses, rushes, and sedges (Bacon and White 2000; Khiralla et al. 2017). Many of its members produce alkaloids, which are toxic to animals and humans, whereas nonclavicipitaceous endophytic fungi, mainly in association with leaves of tropical trees, have been discovered to play an important role in defending the host from abiotic stress, fungal pathogens, and an increase in the biomass (Fröhlich and Hyde 1999; Gamboa and Bayman 2001; Khiralla et al. 2017; Yadav and Yadav 2018; Yadav 2019)

Endophytic fungi play vital roles in host plants, protecting them from stress conditions, making nutrients, such as phosphorus, potassium, and many more,

available, producing auxins, cytokinins, gibberellins, siderophores, ammonia, HCN, and diverse hydrolytic enzymes, and ultimately promoting the growth of host plants. A number of studies suggest that inoculating crops with endophytic fungi might improve growth by diverse plant growth-promoting traits and might also mitigate the effect of stress conditions. Khan et al. (2011b) demonstrated the role of a newly isolated endophytic fungus, *Penicillium funiculosum*, with diverse plant growth-promoting attributes in *G. max* growing under salinity stress. The study revealed that the fungus ameliorated the effect of salinity stress. Kedar et al. (2014) studied the growth promotion potential of *Phoma* sp. isolated from *Tinospora cordifolia* and *Calotropis procera* for maize. The fungal endophytes were found to enhance growth in inoculated maize plants compared with non-inoculated plants. In the study by Rinu et al. (2014), *Trichoderma gamsii* isolated from the lateral roots of lentil with multifarious plant growth-promoting attributes showed its potential in plant growth promotion conducted under greenhouse conditions using two cereals and two legumes, suggesting its potential to be developed as a bioformulation for application under a mountain ecosystem. Yuan et al. (2017) studied the effect of *Penicillium simplicissimum*, *Leptosphaeria* sp., *Talaromyces flavus*, and *Acremonium* sp. isolated from cotton roots with wilt disease caused by the defoliating *Verticillium dahliae* (Vd080). The study demonstrated that all treatments considerably reduced disease incidence and the disease index. The results clearly signified that these endophytes not only delayed, but also led to a reduction in, wilt symptoms in cotton.

In the study by Asaf et al. (2018), *Aspergillus flavus* CHS1, an endophytic fungus, isolated from the roots of *Chenopodium album* with multiple growth-promoting activities, was assayed for its ability to promote the growth of mutant Waito-C rice. The results revealed an increase in chlorophyll content, root–shoot length, and biomass production. Furthermore, the strain was used to evaluate its potential to improve the growth of soybean under salinity stress. Dastogeer et al. (2018) evaluated whether the colonization of two fungal endophytes isolated from wild *Nicotiana* species from areas of drought-prone northern Australia, and a plant virus, yellowtail flower mild mottle virus, could improve water stress tolerance in *N. benthamiana* plants. Inoculation with the fungal strains and the virus considerably increased the tolerance of the plants to water stress. Inoculation with the fungal strains alone resulted in an increase in the relative water content, soluble sugar, soluble protein, proline content, plant biomass, and enzymatic activity, and a decrease in the production of reactive oxygen species and electrical conductivity. Furthermore, there was noteworthy upregulation of numerous genes that had previously been identified as drought-induced. The influence of the virus was similar to that of the fungi in terms of increasing the plant osmolytes, antioxidant enzyme activity, and gene expression. Fungal endophytic communities associated with plants play a vital role in balancing the ecosystem and in enhancing the growth of hosts. They have been shown to be potent biocontrol agents; furthermore, they produce a large number of fungal metabolites that could protect the host from disease, insects, and mammalian herbivores. They have been known to increase the tolerance of their host to abiotic stress.

Thus, fungal endophytes are gaining greater attention and are of greater interest to chemists, ecologists, and microbiologists as a treasure of biological resources, because of their diverse vital roles in the ecosystem.

1.4 Future Prospects and Conclusion

For the previous two and half decades, the scientific community has been aware of the effective role of fungal endophytes in agriculture, ecology, biotechnology, and industry. Fungal endophytes are also an alternative to existing industrial processes of transformation of lignocellulosic biomass, possessing great potential for application in the lignocellulosic industry. The ability of hydrolytic enzymes to synthesize can be employed in enzyme fermentation industries. New techniques with advanced sensitivity are required for enzyme quantification, such as fluorescence spectrophotometry, near-infrared-, and Fourier-transform infrared-based methods. The consequences of enzymes generating endophytes with distinctive consideration of remediating environmental pollutants, such as metals, polyaromatic hydrocarbons, and polychlorinated hydrocarbons, have been understated at the very minimum. Production of secondary metabolites of interest to the pharmaceutical industry is a very attractive field of research using biotechnological methods. The integration of genetic manipulation technology to progress the research to recognize the regulatory gene/s of numerous biosynthesis pathways of metabolite construction can lead to an increase in growth production of the compounds to be used for human welfare. The participation of fungal endophytes in the cycling of nutrients has significant consequences for living organisms and human health. For future research, there are still many areas that need to be explored, including new technologies and new crops with endophytes. Modern techniques of molecular biology, involving metagenomes, proteomes, and transcriptomes, will help to define the characteristics of endophytes and to find novel products for industrial development. The future of research into endophytes is bright, as demand for pharmaceutical products and agricultural produce is increasing day by day with an ever-increasing population.

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Chapter 2
Rhizospheric Fungi: Diversity and Potential Biotechnological Applications

Subha Swaraj Pattnaik and Siddhardha Busi

Abstract In the soil ecosystem, plant-associated rhizosphere represents the most dynamic ecosystem providing a close association between plant root and rhizosphere-associated microbial communities. Among the microbiotas colonizing the rhizosphere, rhizospheric fungi hold prominent position but are less explored than that of rhizospheric bacteria. The majority of rhizospheric microbiota, especially rhizospheric fungi, constitutes a complex interface that utilizes the nutrients released by host plant and sets up a platform for the complex interaction between plant, soil, and inhabiting rhizospheric fungi for ecosystem functioning and environmental sustainability. Rhizospheric fungi exhibit a wide range of applications in the field of biomedicine, pharmaceuticals, industries (particularly textile and food processing industries), and agriculture for maintaining a stability of the ecosystem functioning and environmental sustainability. The advent of high-throughput molecular tools and next-generation strategies in genomics and proteomics such as metagenomics, metaproteomics, metatranscriptomics, and metabarcoding has revolutionized the complete understanding of the widespread potential of rhizospheric fungi. In addition, these advanced tools also provide an insight into the structure, function, and composition of rhizospheric fungi, their untapped ecosystem services to the welfare of human beings and environment, and widespread and untapped biological activities. The intervention of next-generation sequencing methods and chip-based technologies also seeks considerable attention from the scientific community for target-oriented exploration of rhizosphere-associated fungal community for the service of human healthcare system, industrial applications, ecosystem functioning, and environmental sustainability.

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2.1 Introduction

The soil represents one of the most dynamic and complex ecosystems in the world inhabited by a diverse group of microbial consortia in close association with roots of different plants and are responsible for occurrence of variety of biological and physiological processes (Choudhury and Jain 2012). The soil provides a highly complex thermodynamic platform for the complex interactions between inhabiting microbiota and plant roots thereby contributing to the widespread ecosystem services such as regulation of atmosphere, nutrient recycling and conservation, soil fertility, plant productivity, disease mitigation, heavy metal biosorption, and overall ecosystem stability (Birge et al. 2016; Zhang et al. 2017). Soil represents one of the most dynamic ecosystems and accounts for majority of physiological and biogeochemical processes, confined to a particular region termed as hotspots. The soil hotspots can be broadly grouped into four categories such as rhizosphere (root exudates and rhizodeposits), detritusphere (recalcitrant organic compounds), biopores (processing of recalcitrant organics), and aggregate surfaces (leached out substances from detritusphere) (Kuzyakov and Blagodatskaya 2015). The phenotypic and genotypic diversity of plants critically influence the physicochemical properties of soil thereby contributing to the shape of soil-microbial communities and environmental stability (Purahong et al. 2016; Yadav et al. 2018a). Soil constitutes one of the most vibrant and dynamic reservoirs of microbial diversity living in close association with plants.

The rhizosphere, a belowground root-affected soil portion, represents a unique ecological niche for diverse range of microbiota such as nematodes, bacteria, actinobacteria, and fungi living in close proximity with the root system for essential ecosystem services. The rhizosphere harbors such a large diversity of prokaryotes and eukaryotes that the collective genome of the rhizospheric microbial community is larger than that of the host plant species and hence referred to as the plant's second genome. The microbial communities maintain the social interactions with the root system through high profile chemical communication signals secreted by roots into the rhizosphere plane (Berendsen et al. 2012; van Dam and Bouwmeester 2016; Saleem et al. 2018; Verma et al. 2016; Verma et al. 2017b; Yadav et al. 2018b). The present chapter deals with diversity of different groups of fungi associated with root of diverse plant and their industrial applications in diverse sectors.

2.2 The World of Rhizosphere

Based upon the involvement of different factors in any plant-microbes interaction, the composition of microbial community is significantly different and distributed into three distinct ecological niches such as phyllosphere, endosphere, and rhizosphere (Yadav 2009; Rossmann et al. 2017; Suman et al. 2016). The soil ecosystem comprises of one of the most dynamic and complex zone of ecological niche

surrounding and influenced by plant root system called as rhizosphere. The term "rhizosphere" was coined for the first time by Hiltner way back in the early twentieth century which represents a hot spot for numerous microorganisms. The rhizosphere provides platform for colonizing microorganisms and their physiological and metabolic activities in relation to the root system for plant nutrition, growth and development, carbon sequestration process, nutrient recycling, and ecosystem functioning (Berg and Smalla 2009) (Fig. 2.1). The rhizosphere represents a mesotrophic environment for the growth and development of different organisms such as eubacteria, archaebacteria, viruses, fungi, protozoa, algae, and arthropods (Mendes et al. 2013). The structure and composition of rhizospheric environment are greatly affected by rhizodeposition products such as root exudates, mucilage, secretory products, and respiratory CO_2 (Gunatilaka 2006). The root exudates is composed of sugars, amino acids, proteins, and fatty acids and drives the shape and dynamicity of rhizosphere by attracting microorganisms of soil to colonize the rhizospheric niche directly or indirectly for different ecosystem services (Jambon et al. 2018). The rhizosphere is classified into three intricately correlated zones such as the innermost endorhizosphere, consisting of cortex and endodermis, the middle

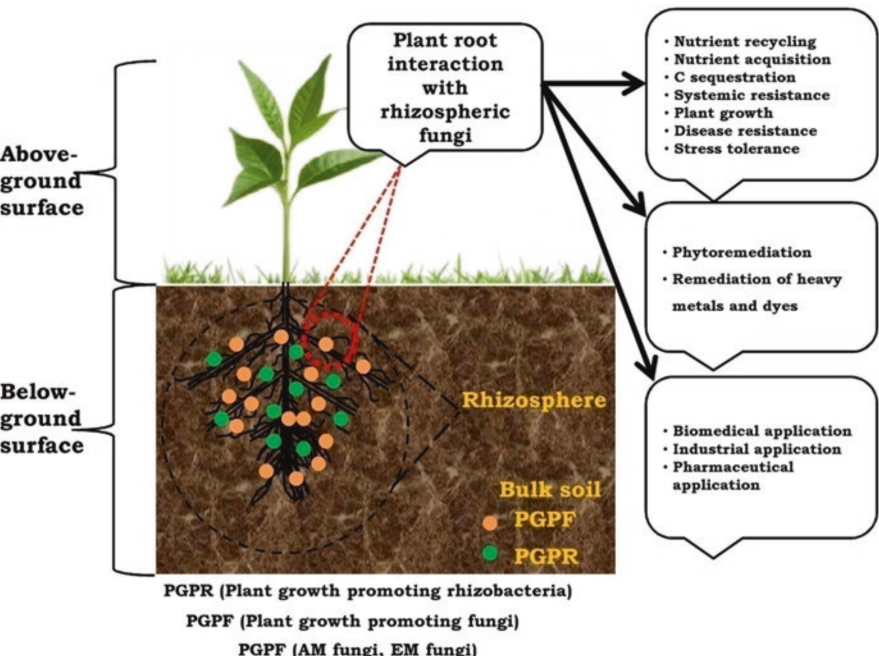

Fig. 2.1 A schematic representation of plant-associated rhizosphere and its associated microbiota. The rhizosphere-associated fungi (AM fungi, EM fungi) in relation to plans exhibit widespread applications in the field of agriculture, pharmaceutical and biomedical industries, and industrial sectors

rhizoplane lying adjacent to root epidermis and mucilage, and the outermost ecto-rhizosphere extending into the bulk soil.

The plant root system and the different gradients of chemical, biological, and physical properties of roots greatly affect the structure, function, and complexity of the rhizosphere (McNera Jr. 2013). The rhizosphere microbiome forms a complex interface in the rhizosphere utilizing the nutrients secreted and released by the plant communities through the root system and sets up a platform for the complex interaction among the host plant, soil, and inhabiting microbial community in such a way to achieve widespread ecosystem services and environmental sustainability (Dessaux et al. 2016). The rhizosphere and the rhizoplane are ecologically important in enhancing the structure, function, composition, and dynamics of colonized rhizospheric microbiota which have the ability to access plant root-associated exudates and participate in shaping growth and agricultural productivity (Poole 2017; Yadav et al. 2017a). The rhizosphere also accounts for different aspects of close interactions such as among the plants and among the microbes and symbiotic interaction between microbes and associated plant. Among these different types of interactions in the rhizospheric interface, the plant-microbes interaction holds considerable importance (Mommer et al. 2016). The plant-associated rhizosphere accounts for a series of collective processes termed as "Rhizosphere effect," occurring at the interface of rhizosphere between the root and soil of a plant. The rhizosphere effects include the root exudation, physiological and metabolic activities of associated microbial communities, exchange of genetic materials between the symbiotic partners, nutrient transformation, and gradient diffusion (Haldar and Sengupta 2015).

2.2.1 Functional Importance of Rhizosphere

The rhizosphere expresses one of the most dynamic and vibrant ecosystems surrounding the roots, where the roots greatly influence the inhabiting microorganisms and in turn rhizospheric microorganisms determine the growth, development, and physiological processes of associated plant thereby presenting a highly evolved interface for intense symbiotic association (Lakshmanan et al. 2014; Shi et al. 2016). The intricate correlation between the rhizospheric microbial community and associated plant roots significantly influences different physical, chemical, and biological processes. In the soil ecosystems, the coexistence of microbial community in association with plant root system ascertains biodiversity sustainability, carbon sequestration, and nutrient recycling in natural and agricultural ecosystems. The plant-rhizosphere interaction significantly affects the metabolic and physiological activity of rhizospheric microbial community and also affects the plant growth, development, and health system and ecosystem functioning (Wang et al. 2017; Yadav et al. 2018a). The rhizosphere also constitutes the soil organic matter (SOM) where majority of carbon is stored and drives the essential elements for primary production by the associated plant, thereby driving the ecosystem functioning and

nutrient recycling (Finzi et al. 2015). The multifaceted application of rhizospheric microbial community and their interactions with associated plants for ecosystem stability and sustainability has propelled the scientific community to develop proper management system for its exploration toward widespread applications for human welfare and environmental sustainability (Mommer et al. 2016; Yadav et al. 2017a).

2.2.2 Composition of Rhizospheric Microbiota

The rhizosphere constitutes an important and metabolically dynamic interface for interaction between plant, soil, and rhizosphere-associated microbial community and allows an interactive exchange of energy and substances. The community structure and composition of the rhizosphere microbiome are greatly affected by many factors such as physical and chemical properties of soil, background microbial composition of soil, plant developmental stage, and plant genotype apart from the environmental conditions (Qiao et al. 2017). Apart from this, the rhizospheric microbial community significantly differs from each other on the basis of different host species as well as different genotypes within a particular species (Jiang et al. 2017). The complexity of soil, soil pH, and soil nitrogen level also potentially determine the structure and function of microbial community in the soil especially in the rhizosphere (Tkacz et al. 2015; Pivato et al. 2017). According to estimates, plants have the ability to release a portion (5–25%) of fixed carbon into the rhizosphere in the form of exudates composed of simple amino acids, organic sugars, fatty acids, and complex mucilages through the root system. The root exudates have a rich lineage of altering the structure and composition of rhizospheric microbial community due to their unique diversity found in different set of plant hosts (Kawasaki et al. 2016). The root exudation process is also greatly affected by a number of abiotic and biotic environmental factors and thereby significantly determines the shape and composition of the colonizing rhizospheric microbiota (Fig. 2.2). The plant host and associated rhizospheric microbial community are intricately correlated with each other at different trophic level and function as an inseparable and unified meta-organism/meta-symbiont or holobiont in the ecosystem with widespread ecosystem services (Bandyopadhyay et al. 2017).

In the rhizospheric ecosystem, both beneficial and harmful microbial community coexists and determines the composition and functional importance of rhizosphere. Mycorrhizal fungi and rhizobacteria symbolize the beneficial microbes in the rhizosphere and coexist in symbiotic relationship with the host plant. The beneficial microbial community provides essential nutrients to the host plant and aids in the photosynthesis and thereby enhancing productivity suppress disease conditions and stress mitigation (Philippot et al. 2013; Rossmann et al. 2017; Mathur et al. 1999).

The structure, function, and diversity of rhizospheric microbial community are directly/indirectly dependent upon the nutrients released from the plants through the root system such as exudates, border cells, and mucilage (Mendes et al. 2014). The rhizospheric microbial community is structurally and functionally different from

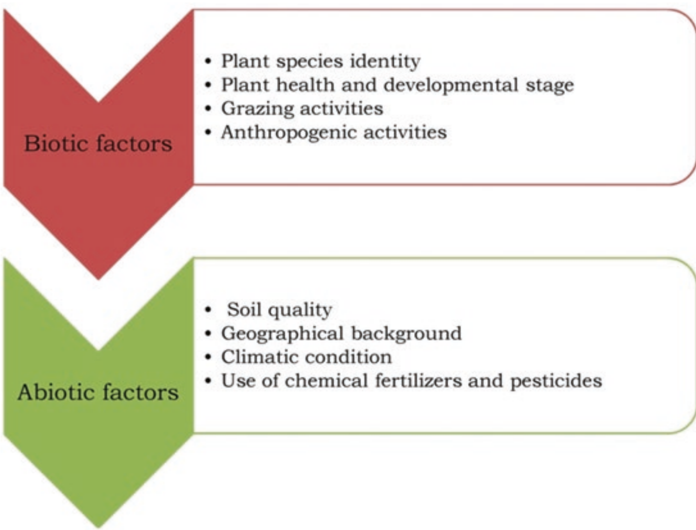

Fig. 2.2 An overview of the different biotic and abiotic factors responsible for determining the structure, shape, and dynamics of rhizosphere-associated microbiomes

the microbial community of adjacent bulk soil due to the presence of root exudates which enables high availability of nutrients and thereby affects the increased microbial biomass in the rhizosphere (Andreote et al. 2014). The rhizospheric microbial community greatly influences the growth, development, and ecological fitness of their associated host plant, production of bioactive compounds of immense biological applications, and geochemical cycling of minerals for better ecosystem functioning (Buee et al. 2009). Most of the plant-associated bacteria and fungi have the ability to produce species-specific microbe-associated molecular patterns (MAMPs), which are recognized and detected by pattern-recognition receptors present in plants, thereby avoiding host immune response for close association and providing ecosystem functioning and credibility (Finkel et al. 2017).

2.3 Rhizospheric Fungi

The rhizosphere harbors a wide range of microorganisms and macroorganisms including an abundance of diverse saprophytic microorganisms due to the increased input of organic carbon compounds into the soil through the process of rhizodeposition. The role of fungi in soil is extremely important and critical to the proper functioning of soil ecosystem by intricately affecting nutrient recycling, maintaining plant growth and development, and maintaining environmental sustainability (Shivanna and Vasanthakumari 2011; Yadav et al. 2018c). The members from *Ascomycota*, *Zygomycota*, *Basidiomycota*, and *Glomeromycota* are found to be

dominant fungal phyla in rhizosphere soil of many plants (Song et al. 2018). Mycorrhizal associations are ubiquitous in nature and present in majority of land plants. These types of associations are ecologically essential for maintaining the functional importance and dynamicity of the rhizosphere. Mycorrhizae are basically categorized into two classes: endomycorrhizae and ectomycorrhizae. Among these two symbiotic associations, arbuscular mycorrhizal (AM) symbiosis represents the most important and critical category based upon its widespread applications (Badri et al. 2009). Arbuscular mycorrhizal fungi (AM fungi) are a group of rhizospheric fungi, present in symbiotic relationship with roots of majority of land plants, grasslands, and mangroves plants (Rajkumar et al. 2012). The rhizospheric root colonized AM fungi are responsible for maintaining soil fertility, increased nutrient uptake and acquisition, community succession, translocation of essential mineral nutrients for plant growth and promotion by inducing polyphenols production, and maintaining plant systemic resistance by inducing antioxidant status during stress and disease conditions (Ceccarelli et al. 2010; Cui et al. 2018). The AM fungi are widely distributed symbiotic fungi existing from ancient times and are associated with diverse range of plants. AM fungi have the inherent lineage to interact symbiotically with the root of associated host plant to transform soil-derived essential nutrients for plant-derived photosynthetic products and enhancing their solubility (Zhou et al. 2017).

The rhizosphere of natural and anthropogenic ecosystems is inhabited by a plethora of organisms in which fungi constitute a large part of the biomass. Many of these rhizosphere fungi are able to colonize the plant and form different types of mycorrhiza. Apart from that, many plants also harbor non-mycorrhizal root-endophytic fungi which greatly influence the regulation of plant nutrition, growth and development, and resistance to stress conditions (Franken 2012). Ectomycorrhizal fungi (EM fungi) and associated bacteria play crucial role in plant-driven mineral weathering and uptake of mineral-derived nutrients in the rhizosphere, thereby maintaining the soil stability and quality (Balogh-Brunstad et al. 2017). The rhizospheric fungi play a crucial role in the ecosystem functioning of rhizosphere by directly or indirectly mediating a number of ecological processes and are responsible for plant growth and development, resistance to stress conditions, and phytopathogens (Berg et al. 2005).

2.4 Interaction of Rhizospheric Microbiota with Plants

The plant-associated rhizosphere is colonized by a variety of microorganisms. The structure and composition of the rhizospheric microbial community are greatly affected by a number of biotic and abiotic factors. The biogenic factors affecting the composition of rhizospheric microbial community are the developmental stage of the host plant, genotype, or cultivar of associated host plant and plant health. Apart from biotic factors, a multitude of abiotic factors such as soil properties, nutrient status, and climatic conditions also greatly influence the structural and functional

importance of the plant-associated rhizospheric microbial community (Berg et al. 2016; Yadav et al. 2018a). Majority of plant species have the tendency to be in symbiotic relationship with soil fungi especially rhizospheric fungi from ancient times. The coevolution of rhizospheric microbial community along with the host plant greatly influences the metabolic and physiological activities such as enhanced mineral nutrient uptake and acquisition, enhanced growth, development and productivity, water-use efficiency, and systemic resistance to pathogenic determinants (Behie and Bidochka 2014). The structure and diversity of rhizosphere-associated fungal communities and the dynamics of interaction with host plants are not only influenced by various biotic and abiotic environmental conditions but also severely affected by the identity and phenological aspect of host plants (Westover et al. 1997; Becklin et al. 2012).

The plant and the microbial communities associated with rhizosphere strongly influence each other via the secretion and detection of certain signaling compounds. The root exudates such as organic sugars, amino acids, organic acids, and secondary metabolites such as phenolic compounds represent the signals sensed by the associated microbial communities at varied levels. In turn, the rhizospheric microbes also produce specific signals which critically take part in plant growth and development and systemic resistance of plants through a process called priming (Venturi and Keel 2016). The identification and functional importance of rhizospheric microbial communities with respect to their environment are important for understanding their effect on metabolic and physiological activities of associated host plants and bioactive metabolites production. In this context, advent of metabarcoding approach allows precise taxonomical identification and characterization from complex environmental samples and provides new avenues for better understanding of plant-microbes interaction and its ecosystem functioning and sustainability (Abdelfattah et al. 2018).

2.5 Potential Applications of Rhizospheric Fungi

Medicinal and aromatic plants are known to produce diverse range of bioactive metabolites with immense therapeutic properties against several forms of diseases. The production of such bioactive metabolites is directly dependent upon the health quality of medicinal plants which concomitantly affected by several biotic and abiotic factors of the rhizosphere. The rhizosphere fungi play a significant role in enhancing medicinal properties of medicinal plants and thereby indirectly affect the production of bioactive compounds of pharmaceutical and industrial importance (Table 2.1). In addition, the presence of rhizospheric microbes plays a crucial role in maintaining agroecosystem sustainability by critically managing nutrient uptake and acquisition, stress tolerance mechanism, and plant growth promotion (Biswas et al. 2018; Verma et al. 2014; Verma et al. 2015; Yadav et al. 2016b; Shaikh and Mokat 2018).

Table 2.1 The rhizosphere-associated fungi and their potential applications

Rhizospheric fungi	Associated plant (root) host	Application	References
Aspergillus effuses H1-1	Mangroves plant	Cytotoxic activity	Gao et al. (2013)
Aspergillus niger	*Azadirachta indica*	Production of antimicrobial compounds	Rani et al. (2017)
Aspergillus sp.	*Phaseolus vulgaris* L.	Phosphate solubilization	Elias et al. (2016)
Aspergillus sp. NPF7	*Triticum aestivum*	PGP	Pandya et al. (2018)
Beauveria bassiana	*Zea mays* L.	Insecticide	McKinnon et al. (2018)
Glomus intraradices	*Echinacea purpurea* L.	PGP	Araim et al. (2009)
Metarhizium anisopliae	*Manihot esculenta* Crantz	PGP and biological control	Greenfield et al. (2016)
Penicillium sp.	*Panax notoginseng*	Cytotoxic activity	An et al. (2016)
Penicillium sp.	*Tithonia diversifolia*	P-solubilization, PGP	Tam et al. (2016)
Penicillium sp.	*Arachis hypogaea*	PGP and stress management	Radhakrishnan et al. (2013)
Penicillium sp.	*Helianthus annuus* L.	Biotechnological applications (inulinase activity)	de Souza-Motta et al. (2003)
Phoma sp.	*Panax notoginseng*	Biological control	Miao et al. (2016)
Phoma sp. MF13	Mangrove sediments	Food processing industries	Wu et al. (2018)
Phomopsis sp. (HKI0458)	*Hibiscus tiliaceus* (L.)	Plant protection from environmental stress	Zhong-Shan et al. (2009)
Piriformospora indica	Colonizes members of bryophytes, pteridophytes, gymnosperms, and angiosperms	PGP and stress management	Varma et al. (2012)
Pochonia chlamydospora	Rhizosphere of plants belonging to Solanaceae and Gramineae family	Anti-nematodal activity and improves host plant defence	Manzanilla-Lopez et al. (2013)
Rhizopus oryzae	*Oryzae sativa*	Biomineralization	Das et al. (2012)
Saccharomycetales sp.	*Panicum antidotale*	Antibacterial properties	Nasim et al. (2018)
Trichoderma hamatum LU592	*Pinus radiate*	PGP	Hohmann et al. (2011)
Trichoderma viride	*Cymbopogon citratus*	Pharmaceutical application	Shaikh and Mokat (2017)

2.5.1 Agricultural Applications

AM fungi have the rich lineage of developing extensively deep into the roots of associated host plant by forming a highly extended extraradical network which helps the associated host plants considerably in exploiting mineral nutrients and water from the soil, thereby maintaining soil health as well as plant growth and development (Jeffries et al. 2003). The root colonization by microscopic soil-borne glomeromycotan rhizospheric fungi, AM fungi greatly influence the crop productivity, enhanced nutrient uptake, nutrient mineralization, nutrient recycling, plant growth and development and tolerance to different environmental stress conditions by intriguingly involved in the plant secondary metabolic pathways (Araim et al. 2009; Cozzolino et al. 2013). Apart from plant growth and development, AM fungi have the inherent tendency to protect the host plants from oxidative stress conditions by modulating antioxidant enzyme machinery and nonenzymatic antioxidant machinery such as glutathione, ascorbate, and α-tocopherol, thereby providing an alternative and effective strategy to counteract the limitations associated with the use of hazardous agricultural chemicals, synthetic fertilizers, synthetic pesticides, and agroecosystems destabilizing fertilizers (Khalid et al. 2017). The agricultural sustainability through rhizospheric microbes could be attributed to their efficacy in solubilizing micronutrients phosphorus, potassium, and zinc (Verma et al. 2015; Verma et al. 2017a; Yadav et al. 2017a; Yadav et al. 2016a; Yadav et al. 2017b) and induce production of the plant growth hormone indole acetic acid (Mwajita et al. 2013; Yadav 2017; Yadav et al. 2018d). The close association between plant root and rhizospheric fungi seems to be the potential determinant of plant health and soil fertility by enhancing the phosphate solubility in the soil by the host plant. Among the rhizospheric fungi, *Penicillium* and *Aspergillus* spp. are the dominant P-solubilizing filamentous fungi with other biotechnological applications such as biocontrol, biodegradation, and phosphate mobilization (Elias et al. 2016). The inherent ability of rhizospheric fungi to solubilize phosphorous and other essential micronutrients has been critically oriented toward enhanced crop productivity under a wide range of agroecosystem, thereby providing candidature for use as biofertilizers (Khan et al. 2010).

Yeasts are one of the important members of eukaryotic microfungi distributed widely in the natural environment. Though yeast system has been defined for various biomedical, industrial, and pharmaceutical applications, their role in management of soil-based agroecosystem still remains undefined. In this context, the plant growth-promoting activities of phosphate-solubilizing yeasts (PSY) *Meyerozyma guilliermondii* CC1, *Rhodotorula mucilaginosa* CC2, and *M. caribbica* CC3 were evaluated and inferred significant enhancement in growth and development of the economically important crop maize (*Zea mays* L.) under field conditions (Nakayan et al. 2013). The species of the genera *Trichoderma*, *Penicillium*, *Fusarium*, and *Phoma* colonizing the rhizosphere are regarded as plant growth-promoting fungi (PGPFs). PGPFs have the ability to influence the plant immune response by intriguingly affecting the induced systemic resistance (ISR) against different pathogenic

determinants in the form of fungi, bacteria, viruses, and nematodes. In addition, PGPFs also significantly promote growth and development of associated host plants and economically important crops. Several PGPF isolates are known for their ability for solubilization of essential nutrient and trace elements which contribute an important role in plant growth, development, and systemic resistance. The main source of resistance induced by PGPF-treated plants is associated with the biosynthesis of defence-related enzymes such as peroxidase and glucanase and other antioxidant enzymes (Jogaiah et al. 2013).

Apart from AM fungi and its application in nutrient uptake and acquisition, some non-mycorrhizal species also come into the picture during nutrient depletion conditions where they have specific mining strategies to provided essential nutrients to the associated plants, thereby promoting plant growth promotion and soil quality. However, more focused and target-oriented approaches are required to have a better understanding of the non-mycorrhizal fungal communities and their association with plants for ecosystem functioning and sustainability (Almario et al. 2017).

2.5.2 Pharmaceutical Applications

Apart from agricultural importance, root colonization with AM fungi in medicinally important plants is also seems to be very important in synthesizing pharmaceutically important metabolites with widespread applications (Johnson and Stephan 2016). Mangroves represent one of the most dynamic and undefined ecosystem harboring a large number of fungal communities. Fungi colonizing the mangrove sediments play an important ecological role in decomposition of organic matter by producing a variety of extracellular enzymes such as amylase, cellulase, pectinase, and xylinase. Such enzymes can be isolated from the mangrove sediment-derived fungi and exploited for a number of industrial and biotechnological applications. Several mangrove fungi-derived bioactive metabolites are also exploited for immense therapeutic, pharmaceutical, and nutraceutical properties. In addition, mangrove fungi also contribute substantially toward production of nontoxic and eco-friendly biopesticides useful in control of plant diseases. Some mangrove-based fungi are also exploited for synthesis of microbial lipids for bioenergy production (Thatoi et al. 2013).

2.5.3 Biomedical Applications

Plant parasitic nematodes, especially root-associated nematodes have the ability to cause potential damage to agricultural crops and thereby alter the agricultural productivity and soil fertility. To control these root-associated nematodes, chemically synthesized fertilizers are extensively used which provide negative results in the long run. In this context, rhizosphere-associated fungal communities are explored

for their efficacy in regulating nematode infections by producing antagonistic nematoxic compounds (Qureshi et al. 2012). Rhizospheric fungal species are known for their ability to synthesize an array of bioactive metabolites with widespread applications. Curvularin and its derivatives are produced by rhizospheric fungal communities belonging to genera *Curvularia*, *Aspergillus*, *Alternaria*, and *Penicillium*. Curvularin and its derivatives exhibited tremendous biological activities such as antifungal properties and cytotoxicity against several human cancer cell lines (Meng et al. 2013). Plant-associated rhizospheric microorganisms especially rhizospheric fungi represent untapped resources of small-molecule natural products with high chemical diversity and could be exploited for biological and ecological relevance. The presence of the bioactive metabolite, monocillin I in the plant-associated rhizospheric fungus, *Paraphaeosphaeria quadriseptata*, is responsible for providing potent cytotoxicity against different cancer cell lines. From this study, the target-specific genome mining approach for large-scale production of monocillin I for benefits of human healthcare could be carried out (Paranagama et al. 2007).

Alkaloids are one of the important classes of phytoconstituents in plants with the potential to be used as effective biocontrol agent. Apart from that, alkaloids have tremendous pharmacological and biomedical applications (Patel et al. 2012). The presence of prenylated indole diketopiperazine alkaloids, isolated from mangrove rhizosphere-derived fungus, *Aspergillus effuses* H1–1, exhibited potent cytotoxic effects against different human cell lines suggesting its candidature for widespread biomedical application to treat life-threatening tumors and cancers (Gao et al. 2013). The entomopathogenic fungus *Beauveria bassiana* colonizing the rhizosphere of maize (*Zea mays*) has the ability to control insect population and has been explored as an effective biopesticide and biocontrol agent (McKinnon et al. 2018). The advent of myconanotechnology seems to be an emerging field, where fungi can be harnessed for the synthesis of eco-friendly and biocompatible nanomaterials owing to their ability for the reduction of metal compounds into their respective nanoparticles by specific enzymes. The biogenic and green chemistry approaches for the synthesis of nanoparticles have gained considerable attention in recent years for biomedical and pharmaceutical applications (Panjiar et al. 2017; Fatima et al. 2015).

2.5.4 Industrial Applications

Fungi represent one of the most diversified groups of microorganisms with widespread applications in the field of agriculture as bioinoculants, in biomedicine for therapeutics, and in industrial sectors as biocatalyst. The absorptive mode of nutrition in fungal community has resulted in the evolution and secretion of arsenal of different enzymes such as amylases, cellulases, proteases, and lipases that catabolize complex organic polymers present in the environment to be absorbed by their cells for metabolic and physiological activities. Fungi accounts for majority of industrial enzymes used as biocatalyst in different industrial setup such as food

manufacturing and processing industries and bioremediating agents in textile-based industries (Suryanarayana et al. 2012). Fungal-derived enzymes show immense physicochemical properties such as high catalytic efficacy, increased substrate specificity, shorter reaction time, low energy input, and mild eco-friendly reaction conditions. Laccases represent one of the biologically important enzymes used for potential industrial application to reduce the environmental pollution and degradation in an eco-friendly approach to maintain the sustainability of the environment. Fungal-derived laccases show widespread applications in synthesis of pharmaceutically important drugs in therapeutics, as stabilization agents in food processing industries, and as effective bioremediating agents for eco-friendly remediation of toxic dyes, pesticides, and heavy metals from environment (Senthivelan et al. 2016).

The use of microbial amylases has successfully replaced the limitations of chemical hydrolysis of starch in starch processing industries. Microbial amylases also have potential application in a number of industrial processes such as in food processing and packaging, textile, and paper industries. Among the fungal species, *A. niger* colonizing the rhizosphere of different plants is a good source of industrially relevant amylase with widespread biotechnological spectrum (Saranraj and Stella 2013).

In the rhizosphere, AM fungi symbiosis could be attributed to plant growth and development by characteristically improving potassium (P) uptake and reducing uptake of metals such as copper (Cu), arsenic (As), and cadmium (Cd). Thus, AM fungi have dual role to play, i.e., plant growth promotion and bioremediation of toxic heavy metals by increasing metal bioavailability at low levels of contamination and in reducing metal bioavailability at high levels of contamination, thereby maintaining a proper balance for ecosystem functioning and environmental sustainability (Kangwankraiphaisan et al. 2013). AM fungi also have the ability to be used as effective phytoremediation strategies to remediate trace elements and heavy metals present in the soil and water bodies, thereby maintaining the quality and stability of soil, providing nutrient uptake to the host plants, and thus managing sustainability in agricultural system (Cabral et al. 2015).

The world is facing a stiff challenge for growing demand of energy on the onset of limitations in fossil resources. In this context, there is an urgent need to explore and develop alternative and renewable sources of energy. In this regard, fungi emerged as an alternative source for biofuel production in the current situation and could be implemented in the near future to achieve the goal of generating biofuel on the onset of depletion of natural resources. Fungal communities are involved for the production of ethanol or future hydrocarbon biofuels and create a range of opportunities in the industrial sectors. However, more advanced genomics studies are required to tune up the efficacy of fungal biomass and their symbiotic association with specific energy crops such as *Miscanthus*, switchgrass, cottonwoods (hybrid poplars), hybrid willows, and sugarcane for achieving the target of renewable energy resources (Grigoriev et al. 2011).

2.6 Current Trends and Future Perspectives

The physiological activities of rhizospheric microbial communities in the rhizosphere present an important aspect in the proper functioning of ecosystem, and hence assessment of structure and composition of rhizospheric microbiota is necessary. Apart from that, it is also imperative to have an insight into the world of rhizospheric microorganisms and their way of maintaining and stabilizing the ecosystem functioning and environmental sustainability (Bakker et al. 2014). Though fungal communities including rhizosphere-associated fungi are being exploited for widespread biomedical, pharmaceutical, industrial, and agricultural applications, till now only 5–10% of all the fungi described are exploited, and a majority of fungal communities are underexplored and remain untapped in the nature's reservoir, which needs to be taken care of for environmental sustainability and human healthcare system (de Felicio et al. 2015). As evidenced from earlier studies and reports, the richness and variation in rhizospheric fungal communities are crucial in attaining sustainability in agriculture by improving soil quality and fertility, ecosystem functioning, nutrient acquisition, and dynamics. In this context, insight studies on rhizospheric fungi and their association with roots could provide ample opportunities and widespread avenues in the quest for potent antagonistic microorganisms for use in the suppression of plant pathogens and maintaining systemic resistance (Song et al. 2018). The advancement in biotechnological applications and high-throughput meta-omics technologies have revolutionized our understanding on rhizospheric microbial communities and their close association with plant root in realizing the widespread avenues in synthesizing bioactive metabolites of biomedical and pharmaceutical importance and maintaining environmental sustainability through bioremediation of heavy metals and toxic dyes (Kothari et al. 2016). The advent of metagenomics and metaproteomics approaches to characterize rhizospheric microbial communities will give impetus to identify novel gene clusters and their engineered products for widespread biomedical, pharmaceutical, and industrial applications (Baeshen 2017) (Fig. 2.3). The advent of nanotechnology has revolutionized the field of biology and is extensively applied toward potent biomedical, pharmaceutical, and agricultural applications. The biogenic synthesis of nanoparticles using bacteria as stabilizing agent has been extensively studied, and in recent past synthesis of metallic nanoparticles using fungal materials especially rhizospheric fungi has come into existence. One of the common example of rhizospheric fungi is *Rhizopus oryzae* colonizing the root of rice plants (*Oryzae sativa*), which serves as an important microbial nanofactory for synthesis of metallic nanoparticles such as gold and silver nanoparticles owing to its advantageous properties such as low-cost synthesis, nontoxic, nonpathogenic, and ability to produce significant quantity of proteins as stabilizing material (Das et al. 2012).

In recent years, the development of advanced community profiling techniques such as terminal restriction fragment length polymorphism (T-RFLP), denaturing gradient gel electrophoresis (DGGE), temperature gradient gel electrophoresis (TGGE), single-strand conformation polymorphism (SSCP), amplified rDNA

Fig. 2.3 An overview of high-throughput molecular tools and advanced technologies to study the taxonomic and molecular identification of plant-associated rhizospheric fungi in relation to the plant root system for their potential applications in agriculture, pharmaceutical, and industry

restriction analysis (ARDRA), and amplified ribosomal intergenic spacer analysis (ARISA) seems to be promising to understand the untapped diversity and ecological role of fungal communities associated with rhizosphere of medicinal plants and economically important crops (Anderson and Cairney 2004). The rhizosphere represents one of the most vibrant and dynamic ecological interfaces due to intense microbial metabolic activities in relation to the associated root system mediated by an array of enzymatic processes. The advent of high-throughput pyrosequencing-based approaches and microarray-based strategies provides an insight into the composition of rhizospheric microbial community and their enzyme-mediated metabolic activities (Li et al. 2014). In the first half of the twenty-first century, the advent of high-throughput molecular tools and intervention of omics technologies have provided ample opportunities to exploit genomics, transcriptomics, proteomics, and metabolomics approaches to identify the taxonomical importance of rhizospheric fungal traits and their structure and functional importance for human welfare and environmental sustainability. These advanced molecular approaches successfully manipulate the rhizospheric microbial community in relation to host plant species for enhanced nutrient uptake, nutrient acquisition, plant growth and development, resistance to stress conditions, and degradation of pollutants to achieve proper ecosystem functioning and environmental (Abhilash et al. 2012).

2.7 Conclusion

The rhizosphere occupies a unique hotspot of soil ecosystem and is one of the most dynamic ecosystems in the earth. The rhizosphere holds a unique niche for close interaction of plant root system with the colonizing microbiota. Among the rhizospheric microbiota, rhizospheric fungi hold a prominent position for widespread pharmaceutical and industrial applications, agricultural sustainability, and maintaining ecosystem services. Though rhizospheric fungi were characterized both morphologically and at molecular level for exploring its potential for service of mankind and ecosystem, majority of rhizospheric fungi are still undefined and present an untapped reservoir for widespread avenues. The advent of high-throughput molecular tools, biotechnological applications, metagenomics and metatranscriptomics strategies, genome mining approaches, and metabarcoding strategies to define the current issues and to discover the untapped potential of rhizospheric fungi has provided the scientific community to understand and work toward sustainable use of rhizospheric fungi in the near future.

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Chapter 3
Trichoderma: Biodiversity, Ecological Significances, and Industrial Applications

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Abstract The genus *Trichoderma* is ubiquitous in the environment, particularly in soils. *Trichoderma* species could be readily isolated from soil by all available conventional methods, largely because they grow rapidly and also because of their abundant conidiation. Based on the phylogenetic study, several researchers reported that *Trichoderma* and *Hypocrea* form a single holomorph genus, within which two major clades can be distinguished. The species of *Trichoderma* possess diverse

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biotechnological applications such as they act as biofungicide for controlling various plant diseases, as biofertilizers for plant growth promotion. *Trichoderma* secrete diverse volatile compounds including alcohols, aldehydes and ketones, ethylene, hydrogen cyanide, and monoterpenes, as well as nonvolatile compounds including peptaibols and diketopiperazine-like gliotoxin and gliovirin which are known to exhibit antibiotic activity. The interaction of *Trichoderma* with the host plant results in parasitism/predation; production of antibiotic is combined with mycoparasitism (penetration and infection), production of cell wall-degrading enzymes or lytic enzymes, competition for nutrients or for space, and establishment of induced resistance in the plant.

3.1 Introduction

The species of *Trichoderma* are cosmopolitan soil fungi significant for their rapid growth. These are capable of utilizing different substrates and are known to be resistant to various toxic chemicals. They are known to be dominant in diverse soils including agricultural, forest, salt marsh, and desert soils in all climatic zones (Kubicek et al. 2003; Wardle et al. 1993). Furthermore, *Trichoderma* species are known to be abundant on decaying wood and in soil because of their successful heterotrophic interactions, such as parasitism, decomposition, and even opportunistic endophytism. Adding more, certain species of *Trichoderma* have been used as biocontrol agents against other phytopathogens and also for the production of enzymes (Samuels et al. 1994). At present this genus consists of more than 260 species (Bissett et al. 2015; du Plessis et al. 2018), and about 35 established species of *Trichoderma* are of economic importance either because of their ability to produce enzymes and antibiotics or use as biocontrol agents (Hjeljord and Tronsmo 2005; Kubicek et al. 2003).

Trichoderma sp. has been known since the 1920s for their capability to act as biocontrol agents against plant pathogens. The principal mechanisms for biocontrol have been assumed to be antibiosis, mycoparasitism, and competition for resources and space (Verma et al. 2017a). Current advances reveal that the effects of *Trichoderma* on plants, including induced systemic or localized resistance, are also very important (Harman et al. 2004). *Trichoderma* sp. and rhizobacteria including species of *Bacillus*, *Enterobacter*, *Pseudomonas*, *Streptomyces*, and others have evolved many mechanisms that improve plant resistance to disease, plant growth, as well as productivity (Wang et al. 2000; Yadav et al. 2018a, b, c; Yadav and Yadav 2018). The present chapter describes the biodiversity of a ubiquitous fungus *Trichoderma* from diverse sources and its applications in industry as producer of bioactive compounds and extracellular hydrolytic enzymes and in the agriculture as plant growth prompter and biocontrol agents.

3.2 Biodiversity of *Trichoderma*

The genus *Trichoderma* has been revealed to be associated with plants as epiphytes and endophytes as well as in rhizospheric region. The genus *Trichoderma* consists of globally distributed fungi. *Trichoderma* exists probably since at least 100 millions of years, but it entered the scientific spotlight only in the late 1970s of the last century, when the first oil shock prompted governments to look for alternatives for fossil fuel. *Trichoderma* sp. are highly successful colonizers of their habitats, reflected both by their proficient utilization of the substrate at hand and their secretion capacity for antibiotic metabolites and enzymes. *Trichoderma* plays significant roles in plant growth promotion and soil health. Till date a huge species of *Trichoderma* has been reported. Figure 3.1 shows the phylogenetic profiling of *Trichoderma* species reported from diverse sources worldwide.

Epiphytes are the beneficial microflora that is present on plants. The aerial parts of plants are the habitat of many epiphytic microbes, which may be harmful or beneficial to the plant. Some of these beneficial microbiota may be actively antagonistic thereby protecting the plant from invasion by the harmful organism. But compared to antagonists isolated from soil, aerial antagonists are reported to be less efficient due to obvious reasons inherent in their respective niche. In spite of this, efforts are being made to exploit the potential of natural epiphytic antagonistic microflora for the management of many plant diseases. Alvindia and Natsuaki (2008) reported the antagonistic nature of fungal epiphytes isolated from banana for the management of banana crown rot disease.

Endophyte is derived from the Greek word "endon" which means within and "phyte" meaning plant. Thus the term endophytic refers to interior colonization of plants by bacteria or fungi. Endophytic microorganisms exist within the living tissues of most plant species (Suman et al. 2016; Verma et al. 2016b, 2017b; Rana et al. 2018). Petrini (1991) first defined endophyte as microorganism living in the plant organization for a certain stage of its life and would not cause disease. Wagenaar and Clardy (2001) identified endophytes as microorganisms that grow in the intercellular spaces of higher plants and are recognized as one of the most chemically promising groups of microorganisms in terms of diversity and pharmaceutical potential. *Trichoderma* species are typically considered to be soilborne organisms and are known for their potential to control plant disease (Harman et al. 2004). *Trichoderma* sp. has also been found as endophytes of plants. There are few reports on *Trichoderma* sp. from banana tissues. Early in the year 2000, Pocasangre et al. (2000) surveyed the distribution of endophytic fungi from banana in Central America and isolated a *Trichoderm*a sp. from the central cylinder. Sikora et al. (2008) reported that *Trichoderma atroviride* Bissett was isolated from the endorhiza of bananas and used for the biocontrol of nematodes. In addition to this, there are also systematic studies on composition and distribution of endophytic *Trichoderma* species in banana plants (Photita et al. 2001). A diverse collection of *Trichoderma* isolates has been obtained, and most have been isolated from live sapwood

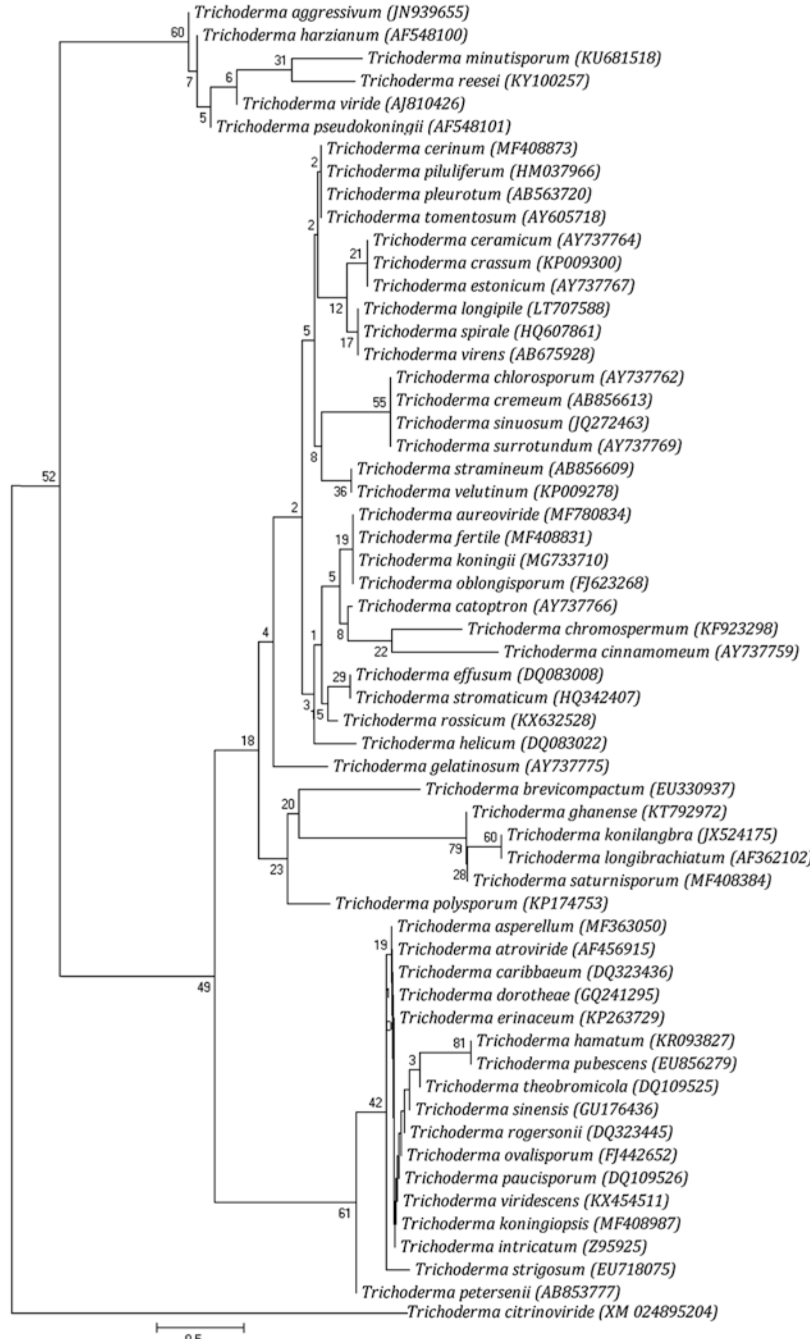

Fig. 3.1 Phylogenetic tree showed the relationship among *Trichoderma* spp. isolated from diverse sources

immediately below the bark of trunks of wild and cultivated *Theobroma cacao* and other *Theobroma* species (Evans et al. 2003).

The approach for making the collection was to look for endophytes coevolved with the pathogens of cacao and its relatives in the upper Amazon region and Choco´ phytogeographic region (Holmes et al. 2004). Only a small number of *Trichoderma* isolates from the collection have been studied in any detail for their biocontrol ability (Samuels et al. 2006a). Abo-Elyousr et al. (2014) identified *Trichoderma harzianum* and *Trichoderma longibrachiatum* associated with onion flora stalks. Cacao trees are vast reserves for endophytic microbial populations (Arnold and Herre 2003; Arnold et al. 2003; Rubini et al. 2005) including many species of *Trichoderma*, some of which are new species. Newly identified endophytic *Trichoderma* species include *Trichoderma ovalisporum* (Holmes et al. 2004), *Trichoderma martial* (Hanada et al. 2008), *Trichoderma stromaticum* (Samuels et al. 2000), *Trichoderma theobromicola*, and *Trichoderma paucisporum* (Samuels et al. 2006b) and *Trichoderma evansii* (Samuels and Ismaiel 2009). Verma et al. (2007b) made a systematic study of the endophytes of *Azadirachta indica*. A total of 233 isolates of endophytic fungi representing 18 fungal taxa were obtained from segments of bark, stem, and leaves of this tree. The dominant endophytic fungi isolated were *Phomopsis oblonga*, *Cladosporium cladosporioides*, *Pestalotiopsis* sp., *Trichoderma* sp., and *Aspergillus* sp. Xia et al. (2011) observed three specific groups of *Trichoderma* in the roots of banana. First group was isolated from the surface of the banana roots, which comprised of *Trichoderma asperellum*, *Trichoderma virens*, and *Hypocrea lixii*, while the second group comprised of *Trichoderma atroviride* and *Trichoderma koningiopsis*, which existed on the surface only, and the third group consisted of *Trichoderma brevicompactum* isolated from the inside of the roots.

Trichoderma sp. was isolated from roots of *Coffea arabica* from the major coffee-growing regions of Ethiopia, and the study revealed that community of *Trichoderma* spp. in roots of *C. arabica* contains fungi both from coffee rhizosphere and putatively obligate endophytic fungi (Mulaw et al. 2013). *Trichoderma theobromicola* and *T. paucisporum* were isolated as an endophyte from the trunk of a healthy cacao tree (*Theobroma cacao*, Malvaceae) in Amazonian Peru and cacao pods partially infected with frosty pod rot, respectively (Samuels et al. 2006b). *Trichoderma citrinoviride* PG87 was isolated from the roots of *Panax ginseng* plants in Korea (Park et al. 2018).

Plants live in close association with the microbes that inhabit soil in which plants grow. Soil microbial communities signify one of the greatest reservoirs of biological diversity known in the world so far (Verma et al. 2014, 2015a, b, c, 2016a, b). The rhizospheric region, which is the narrow zone of soil influenced by root secretions, can have up to 1011 microbial cells per gram root (Egamberdieva et al. 2008) and more than 30,000 prokaryotic species (Mendes et al. 2011). This section deals with the diversity of *Trichoderma* species from rhizospheric region.

Trichoderma harzianum (T-12) and *Trichoderma koningii* (T-8) were isolated from rhizosphere soil of bean, maize, tomato, and radish in New York (Ahmad and Baker 1987). Antagonistic *Trichoderma* spp. was isolated from the rhizosphere of groundnut, and its taxonomic identification was done. The identified strains were

Trichoderma viride (GRT-1, GRT-6 and GRT-9), *Trichoderma koningii* (GRT-2, GRT-5 and GRT-8), *Trichoderma* sp. (GRT-3), *Trichoderma reesei* (GRT-4), *Trichoderma harzianum* (GRT-7), and *Trichoderma aureoviride* (GRT-10) (Sekhar et al. 2017). Aziz et al. (1997) isolated *Trichoderma lignorum* from bean rhizosphere to check the influence of bean seedling root exudates on the rhizosphere colonization by *Trichoderma lignorum* for the control of *Rhizoctonia solani*. Forty-two isolates of *Trichoderma* from rice fields in four provinces in the Philippines were characterized using rDNA-ITS1 analysis and universally primed polymerase chain reaction (UP-PCR) (Cumagun et al. 2000). Wuczkowski et al. (2003) isolated 46 strains of *Trichoderma* from rhizosphere of *Populus* and *Salix* forest area located southeast of Vienna (Austria). Kubicek et al. (2003) identified *Trichoderma harzianum*/*Trichoderma inhamatum*, *Trichoderma virens*, *Trichoderma spirale*, *Trichoderma koningii*, *Trichoderma atroviride*, *Trichoderma asperellum*, *Hypocrea jecorina* (anamorph: *Trichoderma reesei*), *Trichoderma viride*, *Trichoderma hamatum*, and *Trichoderma ghanense* from soil samples from Southeast Asia.

Hoyos-Carvajal et al. (2009) isolated *Trichoderma asperellum*, *Trichoderma atroviride*, *Trichoderma brevicompactum*, *Trichoderma crassum*, *Trichoderma erinaceum*, *Trichoderma gamsii*, *Trichoderma hamatum*, *Trichoderma harzianum*, *Trichoderma koningiopsis*, *Trichoderma longibrachiatum*, *Trichoderma ovalisporum*, *Trichoderma pubescens*, *Trichoderma rossicum*, *Trichoderma spirale*, *Trichoderma tomentosum*, *Trichoderma virens*, *Trichoderma viridescens*, and *Hypocrea jecorina* (anamorph: *Trichoderma reesei*), along with 11 currently undescribed species from different locations of Mexico, Guatemala, Panama, Ecuador, Peru, Brazil, and Colombia.

Trichoderma koningii was isolated from a take-all suppressive soil in Western Australia which protects wheat against take-all disease and increase grain yield in Australia, China, and the United States (Duffy et al. 1997). *Trichoderma atroviride*, *Trichoderma harzianum*, and *Trichoderma virens* were isolated from rice paddy field habitats in Northern Iran (Kredics et al. 2011). One hundred and forty-six (146) isolates of *Trichoderma* sp. were collected from rhizospheric soils around potato plants in the middle areas of Gansu Province, China (Ru and Di 2012). Kale et al. (2018) identified *Trichoderma harzianum*, *Trichoderma hamatum*, and *Trichoderma viride* from rhizospheric soil of tomato. Nawaz et al. (2018) isolated *Trichoderma hamatum*, *Trichoderma harzianum*, *Trichoderma koningii*, *Trichoderma longibrachiatum*, *Trichoderma longipile*, *Trichoderma pseudokoningii*, *Trichoderma virens*, and *Trichoderma viride* from chilli rhizosphere.

3.3 Biotechnological Applications

3.3.1 Production of Secondary Metabolites

Secondary metabolites are defined as small, organic compounds which are not directly involved in normal growth and reproduction of an organism, but they have important roles in development, signaling, and interaction with other organisms

(Hoffmeister and Keller 2007; Osbourn 2010; Saxena et al. 2016; Yadav et al. 2017a, b). The absence of secondary metabolite does not result in immediate death of the individual, but it result in long-term impairment of the organism's survivability, fecundity, or aesthetics or perhaps in no significant change at all. There are sometimes certain environmental conditions when secondary metabolites are necessary for survival such as siderophores which are required for growth when there are low concentrations of iron (Mukherjee et al. 2012). In case of plants, secondary metabolites play an important role in defense against herbivores and other interspecies defenses, while humans use secondary metabolites as medicines, flavorings, and recreational drugs (Vipul et al. 2014). Whereas Demain and Fang (2000) reported that secondary metabolite are used as a competitive weapons against other bacteria, fungi, amoebae, plants, insects, and large animals used as metal transporting agents and also used as an agent for symbiosis between plants and other organisms. Alkaloids, terpenoids, and phenolics are some important secondary metabolites.

Trichoderma spp. are filamentous fungi (ascomycetes) (Hermosa et al. 2000) that are present in nearly all soils and other diverse habitats. They are well adapted to various ecological conditions and varieties of lifestyles, e.g., in soils, on wood bark, and in many other substrates, and interact with animals and plants. *Trichoderma* species are economically important as they act as biocontrol or biopesticide agents, inhibiting the growth of phytopathogenic fungi. *Trichoderma* act as biocontrol agent due to presence of many extracellular lytic enzymes and secondary metabolites (Cardoza et al. 2007). On the basis of analytical as well as the chemical researches, the species of *Trichoderma* has been recognized to be the prolific producers of numerous secondary metabolites with diverse biotechnological and pharmaceutical importance which include peptaibols, poliketides, pyrones, non-ribosomal peptides, siderophores, terpenes, steroids, polyketides, and nitrogen containing compounds (Müller et al. 2013; Velázquez-Robledo et al. 2011; Vinale et al. 2008a). Currently about 373 diverse molecules have been identified, but in many cases, their specific activity is unknown (Crutcher et al. 2013; Mukherjee et al. 2012). *Trichoderma* spp. has been known to be the well-known producers of peptaibols which are short peptides of non-ribosomal origin and are characterized by the presence of high levels of nonstandard amino acids. Over 700 peptaibol sequences are known to date among which most are produced by *Trichoderma/Hypocrea* (Degenkolb et al. 2008). There are three species of *Trichoderma* which are known to produce several types of secondary metabolites, for instance, the genome of *Trichoderma virens* consists of 440 genes which have been classified (EuKaryotic Orthologous Groups, KOG) as related to the biosynthesis of secondary metabolite, transport, and catabolism. The totals for *Trichoderma reesei* and *Trichoderma atroviride* are 262 and 349, respectively. It has also been revealed that most of the genes for secondary metabolite present in *Trichoderma reesei* are also found in *Trichoderma virens* and *Trichoderma atroviride* (Kubicek et al. 2011a). Secondary metabolites which are produced by *Trichoderma* spp. have been listed in Table 3.1 along with their activities (antimicrobial, antifungal, etc.)

Table 3.1 Secondary metabolites/bioactive compounds production produced by *Trichoderma*

Strains	Bioactive compounds	Effects	References
T. koningii, *T. viride*	Dermadin (U-21, 963)	Antimicrobial activity against *S. aureus* and *Escherichia coli*	Tamura et al. (1975)
T. reesei	Cellulases	Degrade cellulase during root colonization to penetrate the plant tissue	Henrissat et al. (1985)
T. longibrachiatum, *T. pseudokoningii*	Compactin	Act as a cholesterol-lowering agent	Endo et al. (1986)
T. koningii	Koninginin A	Act as a regulator of plant growth	Cutler et al. (1989)
T. longibrachiatum	5-Hydroxyvertinolide	Antagonistic against the fungus *Mycena citricolor*	Andrade et al. (1992)
T. virens	Gliovirin	Antimicrobial against oomycetes and *Staphylococcus aureus*	Howell et al. (1993)
T. longibrachiatum	Bisvertinolone	Antifungal properties	Kontani et al. (1994)
T. harzianum	Fleephilone	Inhibitory activity against the binding of regulation of virion expression (REV)-proteins to REV responsive element RNA	Qian-cutrone et al. (1996)
T. harzianum	Harziphilone	Cytotoxicity against the murine tumor cell line M-109	Qian-cutrone et al. (1996)
T. longibrachiatum	Trichodimerol	Inhibit tumor necrosis factor in human monocytes	Mazzucco and Warr (1996)
T. harzianum	6-(1-pentenyl)-2*H*-pyran-2-one	Antifungal activity	Parker et al. (1997)
T. virens	Trichocaranes A, B, D	Inhibits the growth of etiolated wheat coleoptiles	Macías et al. (2000)
T. viride	Viridepyronone	Antagonistic against *Sclerotium rolfsii*	Evidente et al. (2003)
T. harzianum	Harzianopyridone	Antifungal against *Botrytis cinerea*, *R. solani* and inhibitor of the protein phosphatase type 2A	Kawada et al. (2004)
T. virens	Trichodermamide A	It has a weak cytotoxic effect on three cell lines P388, A-549, and HL-60	(Liu et al. 2005)
T. harzianum	T22azaphilone	Inhibits the growth of *Rhizoctonia solani*, *Pythium ultimum* and *Gaeumannomyces graminis*	Vinale et al. (2006)
T. viride	Emodin	Antimicrobial and antineoplastic agent	Wu et al. (2006)
T. harzianum	Trichosetin	Inhibited the growth of rice, tomato, and medicago	Reino et al. (2008)
T. longibrachiatum, *T. koningii*, *T. viride*	Ergokonin A	Antifungal activity against *Candida* sp.	Reino et al. (2008)

(continued)

Table 3.1 (continued)

Strains	Bioactive compounds	Effects	References
T. virens	Trichodermamide B	Displays cytotoxicity against HCT-116 human colon carcinoma	Reino et al. (2008)
T. virens	Wortmannolone	Inhibitor of the phosphatidylinositol 3-kinase with potential to attack human neoplasms in humans	Reino et al. (2008)
T. virens	Virone	Inhibitor of the phosphatidylinsitol 3-kinase	Reino et al. (2008)
T. virens, *T. viride*	Heptelidic acid	Activity against *Plasmodium falciparum*	Reino et al. (2008)
T. atroviride, *T. virens*	Indole-3-acetic acid (IAA)	Growth and development regulator	Contreras-Cornejo et al. (2009)
T. atroviride, *T. virens*	Indole-3-acetaldehyde	Control root growth in *Arabidopsis thaliana*	Contreras-Cornejo et al. (2009)
T. atroviride, *T. virens*	Indole-3 carboxaldehyde	Induces adventitious root formation in *Arabidopsis thaliana*	Contreras-Cornejo et al. (2011)
T. atroviride, *T. virens*, *T. reesei*	Ferricrocin	Required in the competition of iron in the rhizosphere	Kubicek et al. (2011a)
T. hamatum, *T. viride*, *T. virens*	Gliotoxin	Antiviral, antibacterial, antifungal and immunosuppressive	Mukherjee et al. (2012)
T. harzianum, *T. koningii*	Cyclonerodiol	Inhibits growth of etiolated wheat coleoptiles	Vinale et al. (2012)
T. harzianum	Pachybasin	Biocontrol agent against *R. solani*	Lin et al. (2012)
T. virens	Trichovirin II	Induction of resistance in cucumber plants	Mukherjee et al. (2012)
T. viride	Alamethicin	Induction of plant defense in lima and pathogen resistance	Mukherjee et al. (2012)
Trichoderma spp.	Coprogen B	Solubilize iron unavailable for the plant	Vinale et al. (2012)
T. arundinaceum, *T. harzianum*	Harzianic acid	Antimicrobial, plant growth regulator	Malmierca et al. (2013)
T. brevicompactum	Trichodermin	Fungitoxic metabolite against *Candida* spp.	Shentu et al. (2013)
T. brevicompactum	Trichodermin	Phytotoxic effect	Malmierca et al. (2013)
T. virens	*cis- and trans-ß*-ocimene	Induce expression of JA defense responses-related genes in *Arabidopsis. thaliana*	Crutcher et al. (2013)
T. virens	ß-Myrcene	Regulates the expression of genes (abiotic and biotic stresses)	Crutcher et al. (2013)
T. atroviride, *T. virens*	Abscisic acid (ABA)	Regulates stomatal aperture in *Arabidopsis thaliana*	Contreras-Cornejo et al. (2014)

(continued)

Table 3.1 (continued)

Strains	Bioactive compounds	Effects	References
T. atroviride	Ethylene (ET)	Regulates cell differentiation and defense responses	Contreras-Cornejo et al. (2015)
T. atroviride, *T. harzianum*, *T. koningii*, *T. viride*	6-pentyl-2*H*-pyran-2-one	Antifungal, anti-nematode and plant growth-promoting in tomato and *Arabidopsis thaliana*	Garnica-Vergara et al. (2016)
T. longibrachiatum	Trichokonin VI	Inhibits primary root growth in *Arabidopsis thaliana*	Shi et al. (2016)

Trichoderma spp. is mainly used as a fungal biocontrol agents widely for soilborne diseases, as they are producers of various secondary metabolites as well as extracellular enzymes, including β-glucanase, chitinase, and proteinases (Vipul et al. 2014). For acting as biocontrol agent, they show diverse mechanisms of action in their antagonistic interactions with fungal pathogens such as antibiotic activity, mycoparasitism, competition for nutrients, cell wall-lytic enzyme activity, and induction of systemic resistance to pathogens in plants (Verma et al. 2017b; Yadav et al. 2016, 2018a, b, c). The production of antibiotics by *Trichoderma* sp. is considered to play an important role during biocontrol events. A number of antibiotics as well as antifungal toxins such as trichodermin, gliovirin, and harzianic acid have been known to be produced by species of *Trichoderma* which have a direct effect on other organisms (Singh 2010).

In addition to acting as biocontrol agents, the diverse species of *Trichoderma* have also been used as biofertilizers (Harman et al. 2004). Various formulations using a variety of species of *Trichoderma* are commercially available (Harman 2000). There are two strains of *Trichoderma harzianum* T22 and T39, which are used as active agents in a variety of commercial biopesticides and biofertilizers and are widely applied among field and greenhouse crops for crop production (Vinale et al. 2006). *T. viride*, *T. atroviride*, *T. harzianum*, and *T. koningii* produce pyrone (6-pentyl-2H-pyran-2-one), which is responsible for the release of coconut aroma. Pyrone has shown antifungal activities toward plant pathogenic fungi (Scarselletti and Faull 1994). *Trichoderma* spp. produce cytosporone S, which show in vitro antibiotic activity against some species of bacteria and fungi (Ishii et al. 2013). Many species of *Trichoderma* such as *T. harzianum*, *T. koningii*, and *T. aureoviride* produce koninginins, which has antibiotic activity toward the take-all fungus *Gaeumannomyces graminis* var. *tritici* (Almassi et al. 1991; Ghisalberti and Rowland 1993), and also inhibit the growth of some important soilborne plant pathogens including *Bipolaris sorokiniana*, *Fusarium oxysporum*, *Phytophthora cinnamomi*, *Pythium middletonii*, *Rhizoctonia solani*, and other one viridin produced by the *T. koningii*, *T. viride*, and *T. virens* show in vitro antifungal and phytotoxic activity (Vinale et al. 2014). This secondary metabolite prevents spore germination by fungal pathogens (Howell and Stipanovic 1994; Reino et al. 2008). Adding more, harzianopyridone from *T. harzianum* is known to exhibit antibiotic activity against *Botrytis cinerea*, *Rhizoctonia solani*, *G. graminis* var. *tritici*, and *Pythium ultimum* (Dickinson et al. 1989; Vinale et al. 2006). Vinale et al. (2006) isolated T22azaphilone from liquid culture of *T. harzianum* T22; this secondary

metabolite showed in vitro growth inhibition of several plant pathogens, e.g., *R. solani*, *P. ultimum*, and *G. graminis* var. *tritici. Trichoderma* spp. produce cyclonerodiol and trichocaranes which have shown positive effect on plant development and growth (Macías et al. 2000; Vinale et al. 2012). *T. harzianum* produce harzianic acid which is antibiotic against *Pythium irregulare*, *Sclerotinia sclerotiorum*, and *R. solani* (Vinale et al. 2009). *T. hamatum* produce viridiol, which act as a plant growth inhibitor (Howell and Stipanovic 1994). It reduces the production of mycotoxin, e.g., aflatoxin during the fungal interaction (Wipf and Kerekes 2003) (Fig. 3.2). Thus the application of selected metabolites either to induce host

Fig. 3.2 Chemical structure of secondary metabolites produced by *Trichoderma*

Flaviolin
Tenuazonic acid
Scytalol D
Cerulenin
Neobulgarone D
Neobulgarone E
Chromanes B
Trichocarane B
Trichocarane C
Chromanes A
Macrosphelide A
Glisoprenin C
trans-Fusarinine
Chromanes C
Chaetomin
Coniothyriomycin
Trichocarane A
Coprogen
Coprogen B
Ferricrocin
Trichosetin
Trichocarane D
5-Hydroxy-1-(3-methyl-3-buten-1-ynyl)-7-oxabicyclo [4.1.0]-hept-3-en-2-one
Cyclonerodiol

Fig. 3.2 (continued)

resistance or to promote crop yield will surely be an interesting alternative to chemicals, but a deep study is still needed to carry out regarding nature and fate of mixture of secondary metabolites that are released in the soil.

3.3.2 Production of Antibiotics and Bioactive Compounds

Fungi have the potential to produce toxins (antibiotics) that can kill other microbes, at a low concentration. The diversity of these antibiotics has shown various activities against both prokaryotes and eukaryotes (Saxena et al. 2015; Suman et al. 2015; Yadav et al. 2018c). *Trichoderma* spp. produce antibiotics, and antibiotics production was firstly described by Weindling (1934); but Dennis and Webster (1971) reported the role of antibiosis in the control of plant pathogens. Paracelsin was the first antibiotic secondary metabolite, identified in *Trichoderma* spp. (Brückner et al. 1984). *Trichoderma* spp. produce a large number of compounds with antibiotic activity such as alcohols, aldehydes, ethylene, hydrogen cyanide, monoterpenes, ketones, peptaibols, and diketopiperazine-like gliovirin and gliotoxin. The ability to produce antibiotics by *Trichoderma* spp. is dependent on certain factors, e.g., quantity of microorganism, pH and temperature, and type of substrate. A single *Trichoderma* spp. can produce many antibiotic compounds, and, in a similar way, a given antibiotic can be produced by different *Trichoderma* spp. (Sivasithamparam and Ghisalberti 2014); but study of Luckner (1990) reveals that different isolates of the same species can produce different compounds.

Different strains of the *Trichoderma harzianum* leads to the production of antibiotic, and thus these strains have the ability to reduce wheat take-all (Ghisalberti et al. 1990). Howell and Stipanovic (1983) revealed that antibiotics play an important role in the antifungal activity of *Trichoderma virens*. Marfori et al. (2002) reported that methanolic extract from the dual culture of *Catharanthus roseus* callus and *Trichoderma harzianum* showed significant antimicrobial activity against the *Bacillus subtilis* and *Staphylococcus aureus*. Furthermore, *Trichoderma* spp. which inhabit marine habitats are also known to produce a variety of bioactive metabolites (Ruiz et al. 2013), including the antimycobacterial such as aminolipopeptide trichoderins, antifungal including trichodermaketone A, and cytotoxic such as dipeptide, trichodermamide B, and antibacterial, for instance, tetrahydroanthraquinone and xanthone derivatives (Khamthong et al. 2012). Wu et al. (2014) studied the two new pyridones in the culture of marine *Trichoderma* strain, MF106, and their study reveals antimicrobial effects against human pathogenic strains, such as methicillin-resistant *Staphylococcus aureus* and *Staphylococcus epidermidis*.

Hateet (2017) studied the antibacterial activities of the three compounds, which were isolated from endophytic fungus *Trichoderma* spp., and reported the antibacterial activity of the purified compounds against *E. coli* and *S. aureus.* Study of Awad et al. (2018) has shown that *Trichoderma viride* possess various antimicrobial, antioxidant, anticancer, and antiviral activities. *Trichoderma viride* are considered to be the most promising and effective agents controlling a wide range of microorganisms. *Trichoderma viride* has antifungal effect against *Sclerotium rolfsii*, *Rhizoctonia solani*, *Pythium ultimum*, *Fusarium solani*, and *Candida albicans*, further antibacterial activities against *Pseudomonas fluorescens*, *Escherichia coli*, and *Bacillus subtilis*. Gajera et al. (2016) reported antioxidant effect of *Trichoderma viride* against *Aspergillus niger* Van Tieghem pathogens of collar rot in groundnut.

Vizcaino et al. (2005) studied 24 *Trichoderma* isolates from three section, *Trichoderma* section *Trichoderma*, *Trichoderma* section *Pachybasium*, and *Trichoderma* section *Longibrachiatum* for their antibacterial, antifungal, and anti-yeast activities against a panel of seven bacteria, seven yeasts, and six filamentous fungi. The highest number of strains showing antibacterial and antifungal activities reported was from *Trichoderma* section *Pachybasium*, whereas strains from *Trichoderma* section *Longibrachiatum* showed the highest anti-yeast activities. *Trichoderma* pellets have antifungal activity against *Chondrostereum purpureum*, when it is injected into the trunks of trees and is effective against the silver leaf disease of fruit trees (Dubos and Ricard 1974). *Trichoderma* species is used in the protection of grapevine pruning wounds from infection by trunk pathogens (*Eutypa lata*, *Phaeomoniella chlamydospora* and *Phaeoacremonium* species, *Fomitiporia* spp.) (Mutawila et al. 2016). *Trichoderma* spp. has been demonstrated on a wide spectrum both in vitro and in vivo (Di Marco et al. 2004; Kotze et al. 2011).

3.3.3 Production of Hydrolytic Enzymes

All the economically important crops are damaged by the pathogens, among which fungi is the most aggressive soilborne pathogen, and also has been extremely investigated due to damage the cause to the crops (Yadav 2018; Yadav et al. 2017a; Yadav and Yadav 2018). The chief pathogenic fungal genera include *Botrytis*, *Fusarium*, *Pythium*, and *Rhizoctonia* (Djonović et al. 2007). To control these pathogens, pesticides have been widely used (Gerhardson 2002), but the use of pesticides has resulted in environmental and human health concerns throughout the world (Buès et al. 2004; Punja and Utkhede 2003). Thus, some eco-friendly alternatives are important.

The cell walls of *Trichoderma* spp. are known to produce hydrolytic enzymes, for instance, cellulase, chitinase, etc., which play an important role in biomass degradation (Schuster and Schmoll 2010). Chet (1987) studied that cell wall of phytopathogenic fungi is mainly composed of β-1,3-glucans and chitin, including cellulose in some oomycetes, for instance, *Pythium* spp., but due to the presence of hydrolytic enzymes, *Trichoderma* spp. interfere with the activity of pathogen. Hydrolytic enzymes that are secreted by the *Trichoderma* spp. inhibiting the growth of pathogens such as *Trichoderma harzianum* has been demonstrated to produce hydrolytic enzymes which inhibit the growth of *Crinipellis perniciosa*, which is known to be the causative agent of cocoa (*Theobroma cacao*) disease (Marco et al. 2003). The most important factors for the production of the enzymes by the fungus the most important being the type of carbon source available further, production of hydrolytic enzymes are also dependent on light conditions, growth rate, and secretion stress (Arvas et al. 2011; Martinez et al. 2008; Tisch and Schmoll 2013).

Trichoderma isolates produce hydrolytic enzymes such as chitinases, β-1,3- and β-1,6-glucanases, and proteases (β-1,3-glucan), chitin, or fungal cell walls as the

carbon source (De La Cruz et al. 1993; Elad et al. 1982). *Trichoderma reesei* is the most widely employed cellulolytic producer of cellulose- and hemicellulose-degrading enzymes and is used as a production host for enzymes in industrial applications. The large number of carbohydrate active enzymes produced by *T. reesei* (Häkkinen et al. 2012) forms a complex system that is regulated by a variety of environmental and physiological factors. *Trichoderma reesei* strain QM6 was found to be good producer of hypercellulolytic enzyme, (RUT-C30) (Peterson and Nevalainen 2012), although high levels of cellulase are also produced in other species from this genus (Baig et al. 2003; Watanabe et al. 2006). The use of *Trichoderma harzianum* species in biotechnology has been explored by examining the biocontrol capacity of this species (Liu and Yang 2005; Yao et al. 2013). *T. hamatum* strains possess higher antimicrobial activity due to presence of specific β-glucanase and chitinases, which play important roles as hydrolytic enzymes during cell wall degradation (Cheng et al. 2015). Cheng et al. (2017) studied that YYH13 strain of *T. hamatum* produces cellulase, due to its strong ability to degrade cellulosic biomass. Ahmed et al. (2009) studied the production and purification of three cellulases from *Trichoderma harzianum*: exoglucanase (EXG), endoglucanase (EG), and β-glucosidase (BGL). Small number of cellulases is present in *T. reesei*, *T. virens*, and *T. atroviride*; but all these spp. are enriched in some hemicellulolytic components, such as GH27 α-galactosidases, GH43 α-arabinofuranosidases/β-xylosidases, GH67 and GH79 α-methylglucuronidases and α-fucosidases, cellulase, and xylanase (Kubicek 2013).

Trichoderma reesei produce cellulase, which is used mainly for malting, baking, and grain alcohol production (Galante et al. 2014). Lignocellulosic biomass is used for the production of biofuels, e.g., ethanol (Herpoël-Gimbert et al. 2008; Lin and Tanaka 2006), paper, and textile industries (Galante et al. 2014). *Trichoderma* spp. are also used for industrial enzyme production (Nevalainen et al. 1994), e.g., enzymes are used to improve the brewing process for fruit juice production and as a feed additive for livestock and pet food (Schuster and Schmoll 2010). *Trichoderma* also used for seed germination, for example (Anis et al. 2012), observed significant sunflower seeds germination, on the application of *T. viride* or *T. reesei*. Some commercially available formulations for protection and growth enhancement are RootShieldTM, BioTrek 22TM, T-22GTM, and T-22HBTM, SuprevisitTM, BinabTM, TrichopelTM, TrichojetTM, TrichodowelsTM, TrichosealTM, etc.

Trichoderma spp. are capable of using a wide range of compounds such as carbon and nitrogen sources simultaneously secrete a variety of enzymes to break down plant polymers into simple sugars for energy and growth. Due to high cost of chemical inducers for these enzymes, there is a need to find some cheap organic inducers from agriculture wastes so that mass production of *Trichoderma* species could be increased. Studies on enzymes produced by *Trichoderma* are essential to find more proficient and low-cost enzymes, which will be useful in different steps of the hydrolytic process of biomass degradation.

3.3.4 Role of Trichoderma in Wine Making and Brewery Industry

The main attentiveness of biocatalytic processes is the prospect to develop less adulterating, biodegradable industrial products. A *Trichoderma longibrachiatum* transformant has been fabricated constitutively articulating the homologous egl1 gene. The egl1 product (EGL1) has been purified, which possess both endoglucanase and xylanase activities. Considering the belongings of certain enological factors such as ethanol, glucose, temperature, pH, and SO_2 concentrations on the enzyme's activities reveals that it can be used in wine production (Ganga et al. 1997). A novel S-adenosyl-L-methionine-dependent methyltransferase catalyzing the O methylation of numerous chlorophenols and supplementary halogenated phenols was purified from mycelia of *Trichoderma longibrachiatum*. Studies demonstrated that the doings was also precise for halogenated phenols comprising bromo, chloro, or fluoro, substituents, whereas other hydroxylated compounds, for instance, dihydroxybenzene, hydroxybenzaldehydes, hydroxylated benzoic acids, phenol, and 2-metoxyphenol, were not methylated (Coque et al. 2003).

The stimulus of *Trichoderma* hydrolytic enzymes, with pectinase, cellulase, chitinase, and/or glucanase activities, as well as the supra-extraction of *Palomino fino* grape, was studied for fermentation process, for juice clarification, and for wine characteristic. The uppermost activity was observed with the usage of enzymes in infected juice. Furthermore, the maximum variances in the wine characteristic were perceived when comparing wines from juices subjected to different circumstances (healthy of infected, frozen, or fresh) self-sufficiently of enzymes use. Supra-extracted juices produced wines with improved acidity and higher alcohols such as methanol, propanol, and isobutanol (Roldán et al. 2006).

Trichoderma viride WEBL0703 performed extraordinary level of activity toward a broad spectrum of phytopathogens, which was manufactured by solid-state fermentation using grape marc and wine lees. The yields of some important enzymes which play an important role in protecting plants from various diseases such as chitinase, β-glucanase, and pectinases were 47.8 U/g IDS, 8.32 U/g IDS, and 9.83 U/g IDS, respectively. The study suggested that it is attainable to convert winery wastes to a value-added and eco-friendly biocontrol agent (Bai et al. 2008).

3.3.5 Trichoderma in Agriculture

Trichoderma is one of the utmost studied and functional fungal biocontrol agents. The biological activity is related to the variety of metabolites that they produce which have been found to directly inhibit the growth of the pathogens, enhance disease resistance, and enhance plant growth. *Trichoderma* spp. are well for production of enzymes with high xylanolytic activity. Diverse xylanases have been well characterized, identified, and purified for their physicochemical, hydrolytic, as

well as molecular properties. Cellulase-free xylanase preparations have also been tested successfully in improved or alternative industrial applications (Wong and Saddler 1992).

Biological and chemical control of gray mold was tested in vineyards of table and wine grapes. Treatments with *Trichoderma harzianum* (0.5–1.0 g 1^{-1}), dicarboximide fungicides including vinclozolin or iprodione (0.5 g 1^{-1}), and diethofencarb plus carbendazim (0.25 g 1^{-1}) declined disease up to 78%. *T. harzianum* and iprodione smeared alone in the vineyard led to reduction of postharvest rot of grapes experiments. Oscillation of *T. harzianum* with diethofencarb plus carbendazim, or its mixture with iprodione in the vineyard, reduced the disease by 64–68% in postharvest rot initiated by *Botrytis cinerea* (Elad 1994). Nine transformants of *Trichoderma longibrachiatum* with additional copies of the egl1 gene were observed for endoglucanase production, mitotic stability, and biocontrol activity against *Pythium ultimum* on cucumber seedlings. The study demonstrated that, *Trichoderma longibrachiatum* transformants with improved inducible or constitutive egl1 expression often were more suppressive in comparison to wild-type strain when applied to cucumber seeds sown in *Pythium ultimum* infested soil (Migheli et al. 1998). Biocontrol through the mycoparasitic by *Trichoderma* spp. primarily involves production of cell wall-degrading enzymes.

Economically important cultivated cotton plant was selected to observe the growth progression by *Trichoderma viride* and *Pseudomonas fluorescens* with and without pathogens, *Rhizoctonia solani* and *Macrophomina phaseolina.* Of these, *Trichoderma viride* was found to be more effective than *Pseudomonas fluorescens* on shoot and root length elongation. Seed germination percentage, root length, shoot length, fresh weight, dry weight, and vigor index were significantly increased by *Trichoderma viride* and *Pseudomonas fluorescens* (Shanmugaiah et al. 2009).

Wilt of tomato due to *Fusarium oxysporum* is one of the most severe diseases posturing a threat to crops in fields. Observation of mycoparasitic potentiality of three species of *Trichoderma*, *T. harzianum*, *T. viride*, and *T. hamatum*, toward reducing the consequence of the pathogen on crop was evaluated. All the three tentative species of *Trichoderma* were capable to synthesize lytic enzymes, β-1, 3 glucanase and chitinase, efficiently. *T. harzianum* was observed to be the best in three species of *Trichoderma*. *Trichoderma* in the field showed their ability to decrease the occurrence of the wilt disease to a reasonable level where the *T. harzianum* is superior over the others (Ojha and Chatterjee 2011).

The fungal isolates including *Clonostachys rosea*, *Coniothyrium minitans*, *Trichoderma crassum*, *Trichoderma hamatum*, *Trichoderma rossicum*, and *Trichoderma virens* were tested in two bioassays for their capability to degrade sclerotia and reduce apothecial production and carpogenic infection of cabbage seedlings, when incorporated through soil *Coniothyrium minitans*, and *Trichoderma hamatum* showed potential to control *S. sclerotiorum* disease in cabbage (Jones et al. 2014). The potential for biocontrol of soilborne plant pathogens in *Rhizoctonia solani* (Kuhn), *Pythium ultimum* (Trow), and *Sclerotinia trifoliorum* (Eriks) by *Trichoderma* was observed. Nine *Trichoderma* isolates (five of *Trichoderma atroviride* and one each of *Trichoderma hamatum*, *Trichoderma koningiopsis*,

Trichoderma viride, and *Trichoderma virens*) were selected for valuation in experiments. Seedling progression (shoot and root fresh weight/plant) of these pasture species was expressively improved by one or more *Trichoderma atroviride* isolates. By observing it is found that four *Trichoderma atroviride* isolates were selected for field assessment as biocontrol agents of soilborne pathogens of pasture species (Kandula et al. 2015).

Macrophomina phaseolina remains the prevalent causative agent of charcoal rot disease that considerably suppresses the crops of oilseed. Experiential evidence of the efficiency of three *Trichoderma harzianum* isolates (T2, T10, and T12) as biological control agents against charcoal rot in soybean (Glycine max L.) was evaluated. Isolate T12 of *Trichoderma harzianum* shows extensively higher inhibition effect than T2 and T10 isolates. Therefore, the study supported the applicability of T12 isolate as possible alternate to biocontrol of charcoal rot in soybean (Khalili et al. 2016).

The study of Asmawati et al. (2017) investigated the potency of *Trichoderma* spp. including JMA1, JMA2, KMA, and STA in inducing downy mildew disease and inducing ROS. The production of ROS is one of the earliest cellular responses following successful pathogen recognition. The results revealed that KMA, STA, JMA2, and JMA1 isolates considerably reduced downy mildew disease intensity and induced ROS as a response.

In the study of Pascale et al. (2017), the influence of two *Trichoderma* strains and their secondary metabolites were reported on *Vitis vinifera* in terms of stimulation of antioxidant activity, disease resistance, and plant growth promotion in the grapes. Applications of *Trichoderma harzianum* M10 or *Trichoderma atroviride* P1, as well as their particular major secondary metabolites, harzianic acid, and 6-pentyl-α-pyrone, have been shepherded in greenhouse using foliar spray or drenching. The study revealed that both *T. harzianum* T22 and 6-pentyl-α-pyrone were capable of improving crop yield as well as total amount of polyphenols and antioxidant activity in the grapes

Trichoderma has evidenced potential for its diverse role in agriculture, and numerous strains of *Trichoderma* have been effectively screened out for its constructive effects on soil fertility and plant health aspects, but we need an environment which is free of pollution, and therefore focusing on multiple functions of *Trichoderma* to fight against various biotic and abiotic stresses and the hazardous pollutants which can affect our food chain is important to maintain sustainability (Pal et al. 2017; Yadav 2019). Strains of *Trichoderma atroviride* and *Trichoderma harzianum*, overgrown on biological material, were applied as bio-preparations to the soil in open-field lettuce crop growing, and their inhabitants levels were monitored over time. These *Trichoderma* species were recognized in field soil; however, their abundance was expected to be relatively low 10^3 CFU, when compared to 10^5 CFU g^{-1} of dry soil, subsequently bio-preparations application, and *Trichoderma* continued at this level even after 2 years (Oskiera et al. 2017). Another study employed MiSeq sequencing for the evaluation of the reaction of local microbial communities to three diverse fertilization regimes, including heavy chemical fertilizer application (CF) and reduced chemical fertilizer applications supplemented

with organic (OF) or *Trichoderma*-enriched organic fertilizer (BF) on tomato for five season. The study achieved better plant growth and soil fertility prominence in the BF treatment followed by the OF and CF treatments. The study concluded that as compared to the CF and OF regimes, reduced chemical fertilizer plus *Trichoderma*-enriched organic fertilizer (BF) could be the utmost appropriate regime to control microbiome degeneration of soil and to conserve tomato plant growth and health (Pang et al. 2017).

The influence of grainy organic waste material overgrown with *Trichoderma atroviride* TRS25 on the survival of *Sclerotinia sclerotiorum* and *Chalara thielavioides* in the soil was scrutinized. After the addition of granulated waste material, an increase of bacteria, especially the *Pseudomonas* group in the soil was observed (Kowalska et al. 2017).

3.3.6 Role of Trichoderma in Bioremediation

The species of genus *Trichoderma*, an anamorphic *Hypocreaceae* (class *Ascomycetes*), have been used in the biological control of plant disease, manufacture of cellulolytic and hemicellulolytic enzymes, biodegradation of chlorophenolic compounds, and soil bioremediation (Esposito and Silva 1998; Kour et al. 2019). *Trichoderma reesei* are deliberated to be one of the most efficient hyper producers of cellulase that is used in industry. The biomass concentration as a purpose of time was constant with relatively rapid, early growth on easily metabolized growth medium components (yeast extract), followed by a second slower growth phase due to hydrolysis of cellulose, which follow cellulase concentration augmentation (Ahamed and Vermette 2008). Bioremediation and phytoremediation in association with microbes are pioneer technologies having a prospective to mitigate many environmental problems. The genus *Trichoderma* is genetically very diverse with a numerous capabilities among different strains with agricultural and industrial significance. It is also tolerant to a variety of recalcitrant pollutants such as heavy metals, pesticides, and polyaromatic hydrocarbons. There are vast future prospects of *Trichoderma* for biological or phytobial remediation of environmental contaminants (Tripathi et al. 2013) (Fig. 3.3).

A newly isolated ascomycete fungus *Trichoderma lixii* F21 was explored to bioremediate the polar [Alizarin Red S (Khalili et al. 2016)] and nonpolar [Quinizarine Green SS (QGSS)] anthraquinone dyes. Results revealed that *T. lixii* F21 may be a good candidate for the bioremediation of industrial effluents adulterated with anthraquinone dyes (Adnan et al. 2017). *Trichoderma* species could be a model fungus to sustain crop productivity as well as widely used as inoculants for biocontrol, biofertilization, and phytostimulation. *Trichoderma* species are recounted to increase photosynthetic efficiency, develop nutrient uptake, and proliferate nitrogen use efficiency in agriculture. On the other hand, they can be used to yield bioenergy, facilitate plants for adaptation, and mitigate adverse effect of climate change (Kashyap et al. 2017). *Trichoderma* species have diverse biotechnological

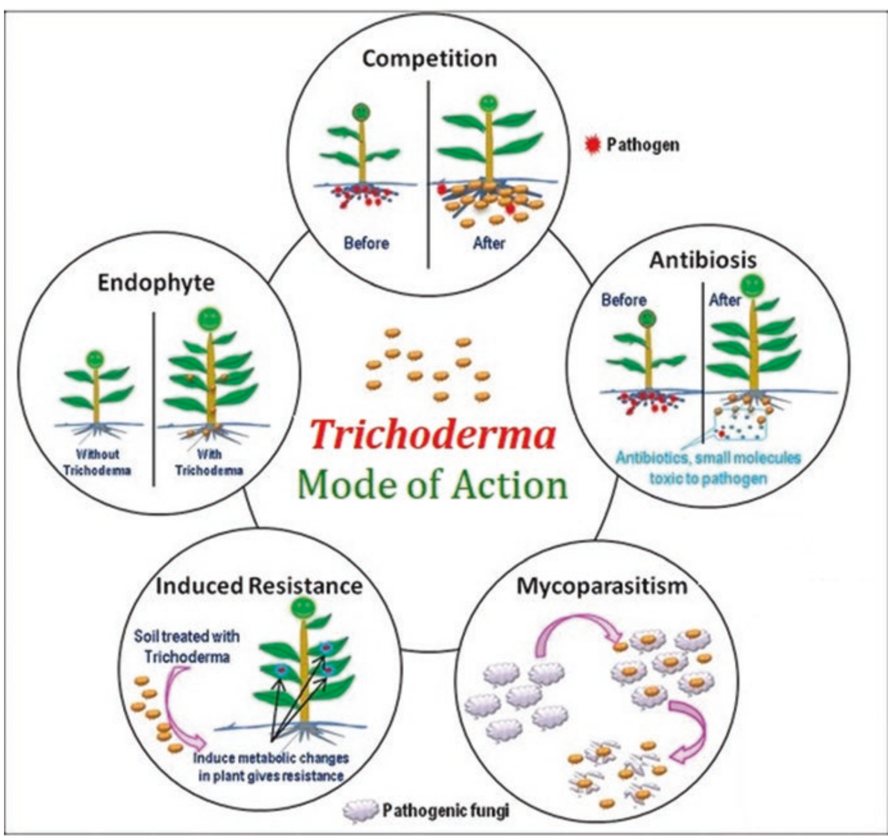

Fig. 3.3 Model depicting mode of action of *Trichoderma* spp. against pathogen and plant growth improvement (Waghunde et al. 2016)

applications as a biofungicide for plant disease control and biofertilizer for plant growth promotory effects resulting in a high yield and productivity ensuring food security, along with environmental security, by reducing the use of hazardous agrochemicals, production of industrially important chemicals, and having a prospective for bioremediation for environmental clean-up activities (Kidwai and Nehra 2017).

Bioremediation using efficient *Trichoderma* can help in eliminating various heavy metals that can pollute the environment. *Trichoderma* was isolated by serial dilution and spread plate techniques on potato dextrose agar (PDA) with an individual heavy metal, i.e., chromium (Cr), copper (Cu), lead (Pb), zinc (Zn), and nickel (Ni). Of the 29 fungal isolates, 4 species were selected, and growth test indicates that all *Trichoderma* isolates can tolerate high levels of Cr and Pb; however, tolerance to Cu, Zn, and Ni was species specific. Results revealed the potential of *Trichoderma* isolates for biological wastewater treatment in mining industries (Tansengco et al. 2018).

3.4 Ecological Significance

3.4.1 *Trichoderma as Human Pathogen*

The genera *Candida*, *Aspergillus*, *Trichoderma*, and *Cryptococcus* are frequently observed pathogenic fungi. Out of these, the genus *Trichoderma was* found in new advancement research as *the* opportunistic human pathogens encountered in human body which are responsible for causing diseases to HIV-infected persons and other immune compromised patients. The geographical distribution of *Trichoderma* is worldwide. They are green-spored, soilborne ascomycetes. On the basis of data obtained from literature, association of *Trichoderma viride* in damp and moldy buildings (caused by excess *moisture)* results in adverse human health effects and are responsible for mucosal/respiratory diseases such as respiratory allergies, asthma, and chronic bronchitis. Inhaled spores from these emerging fungal pathogens reach the lung alveoli, and there they interact with the epithelial lining of the respiratory passages. In all over the world, *Trichoderma longibrachiatum* reported as most common clinical isolates (Robertson 1970; Vicente et al. 2001).

3.4.2 *Trichoderma in Parasitism*

Genus *Trichoderma* is one of the most frequent encountered free-living fungi that are commonly present in rhizospheric soil and root ecosystems. The nature of this genus is parasitic to other phytopathogenic microorganisms and also altruistic symbiont to various plants. They established a long-lasting colonizations with root surfaces and after that it penetrate deep into the root epidermis. If plant roots are colonization by *Trichoderma* spp., the development and growth of root, resistance against abiotic stresses, productivity of crop, and nutrient uptake enhanced (Harman et al. 2004). *Trichoderma* also play a vital role in biological control programs and integrated pest management due to its antagonistic interactions with other fungi or pathogens. Various species are also known to science which produces secondary metabolites with antibiotic properties (Ghisalberti and Sivasithamparam 1991; Reino et al. 2008; Sivasithamparam and Ghisalberti 2014). Trichoderma's interaction with the host plant results in parasitism/predation; the production of antibiotic is combined with mycoparasitism (penetration and infection), production of cell wall-degrading enzymes or lytic enzymes, competition for nutrients or for space, and establishment of induced resistance in the plant (Benítez et al. 2004; Chet et al. 1997; Handelsman and Stabb 1996; Harman et al. 2004; Lorito and Woo 1998; Sivasithamparam and Ghisalberti 2014; Vinale et al. 2008b; Woo and Lorito 2007).

This mode of mycoparasitism means direct attack of one fungus by another ones is necrotrophic resulting in various events such as recognition, attack, penetration, and finally the death of host or pathogenic fungi (Inbar and Chet 1995). An

opportunistic symbiont *Trichoderma* has its cell wall fragments as the initiators of a physiological enzymal cascade within the fungus that also complement its own growth. There are plenty of routes by which *Trichoderma* spp. control or kill other fungi, and for this purpose, three types of interactions are necessary – *Trichoderma*–pathogen, *Trichoderma*–plant, and *Trichoderma*–plant–pathogen interactions (Harman 2006). Mycoparasitism is a complex process that involves chemotropical attachment of *Trichoderma* toward its host and also coiling or penetrating around the host hyphae (Carsolio et al. 1999). *Trichoderma*–pathogen interaction have different pathways and cascade mechanism like MAPKKK, MAPKK and MAPK signaling pathways (Daguerre et al. 2014; Kumar et al. 2010; Reithner et al. 2007). It includes a seven transmembrane G protein-coupled receptor 1; Gpr1 is for sensing the fungal prey in the surroundings (Omann et al. 2012). Direct interaction results in necrotrophic hyperparasitism or mycoparasitism, i.e., the direct attack of one fungus to another fungus (Harman et al. 2004). This complex process involves series of events beginning from cycle of recognition by the binding of carbohydrates in the cell wall to lectins on the target fungus and then hyphal coiling and appressoria formation containing higher amount of osmotic solutes such as glycerol and induction of penetration, attacking on cellular machinery with the help of several fungitoxic cell wall-degrading enzymes like glucanases, chitinases, and proteases (Ragnaud et al. 1984); sum-up action of these compounds results in parasitism of the target fungus and dissolution of the cell walls. The direct entry of *Trichoderma* hyphae into the host lumen and ultimately the death of the host (Kumar 2013).There are at least 20–30 genes, proteins, and other metabolites that are directly involved in this interaction (Daguerre et al. 2014; Singh et al. 2018).

Defense mechanism of *Trichoderma–Trichodermal* spp. introduces different genes like genes for the heat shock response, for oxidative stress response, and for detoxification processes when the prey is around (Lorito et al. 2010; Seidl et al. 2009). Killing the prey: The synergistic action of antifungal secondary metabolites and cell wall hydrolytic enzymes secretion results in killing or death of prey. Most affluent biocontrol agents used nowadays in agriculture are *Trichoderma* spp. with almost 60% of the registered *Trichoderma*-based biofungicides worldwide (Verma et al. 2007a). The consequential restrictions to fungicides (microbe-based) are their inadequate capability and inconsistency.

Microbes are slow in their action as compared to chemicals fungicides and affected by environmental conditions. Genetic intervention is used to design strains that are more successful as compared to the native ones and could be attained by obtaining knowledge regarding the molecular-based mechanisms of interactions of these organisms with biotic and abiotic factors.

According to Chet et al. (1997), Harman et al. (2004), and Heydari and Pessarakli (2010), various mechanisms are used for biocontrol activity of genus *Trichoderma* against phytopathogenic fungi or toward their competitor as given below (a) antibiosis, (b) competition, (c) enzymatic hydrolysis, (d) parasitism, and (e) systemic-induced resistance.

3.4.2.1 Antibiosis

Antibiotics are the secondary metabolites involved in biocontrol or microbial volatile and nonvolatile metabolites toxin that have power to kill other microorganisms at very low concentration. These metabolites results in the production of harzianic acid, alamethicins, antibiotics, peptaibols, tricholin, 6-penthyl-α-pyrone, massoilactone, heptelidic acid, viridian, glisoprenins, and gliovirin (Vey et al. 2001). In vitro and/or in situ condition fungi produce toxins exhibiting activity against prokaryotes and eukaryotes. Many antibiotics originated by microorganisms are effective to many disease caused by plant pathogens (Howell and Stipanovic 1980; Islam et al. 2005; Shanahan et al. 1992; Yoshihisa et al. 1989).

3.4.2.2 Competition

The requirement of the same resource by two or more organisms in an ecosystem and utilization of these resources by one organism reduces the amount availability to the other organism. In the soil and rhizospheric region, nutrient availability is limited for microorganisms as a result of which death occurs due to starvation. For biocontrol agents, there is a belief that competition is required for survival in a particular niche between pathogens and nonpathogens (Elad and Baker 1985; Keel et al. 1989; Loper and Buyer 1991). Sharma (2011) identified the specific genes linked with the mechanism of biocontrol in *T. harzianum* genes encode multidrug-resistant proteins (MDR ProB, MDR Protien2, and MDR Bref A) on the basis of which they are involved in competition for nutrients and for space between two or more organisms.

3.4.2.3 Enzymatic Hydrolysis

Interactions between *Trichoderma* spp. with plant pathogenic fungi result in mycoparasitism in which the antagonist coils pathogen hyphae and develops appressoria that linked with the production of lytic enzymes (Chet et al. 1997; Howell 2003; Kubicek et al. 2011b; Rocha-Ramírez et al. 2002). The host cell wall in the presence of lytic enzymes becomes weak, and in the cell wall, diffusion of antibiotics occurs which increase the concentration of antibiotics (Lorito et al. 1996). Antibiotics and hydrolytic enzymes result in synergism, and cell wall-degrading enzymes (CWDEs), i.e., chitinases [1,4 –β-acetylglucosaminidases (GLcNA cases) endochitinase and exochitinase, glucanases (β-1,3-glucan) and proteases, etc.], are produced for the degradation of cell wall to establish the interaction.

3.4.2.4 Parasitism

Trichoderma spp. are used against various genera of plant–parasitic nematodes or act as a biocontrol agent (Rao et al. 1998; Reddy et al. 1996; Windham 1986). Interactions between *T. harzianum* and *Globodera rostochiensis* (potato cyst nematode) have been demonstrated in vitro. Biocontrol by two different strains of *T. harzianum*, i.e., *T. asperellum*-203 and *T. atroviride* IMI 206040, has been already reported against *M. javanica* in soil (Sharon et al. 1993). In growth chamber experiments, some *Trichoderma* species and their isolates have also showed significant biocontrol activity against *M. javanica* (Spiegel et al. 2007).

3.4.2.5 Induction of Resistance

Each and every plant on this earth respond to various environmental-stimulating factors like against gravity, temperature, light, physical stress, nutrient, water and chemical formed from soil, and microorganisms which live in association with plants. Produced stimuli induce plant host defenses by biochemical changes inside the plant and increases resistance against many infection caused by a variety of pathogens. Induction of this host defenses is systemic or local in their nature related to amount of stimulating agents, type, and source of stimulating agents (Audenaert et al. 2002; De Meyer and Höfte 1997; Kloepper et al. 1980; Leeman et al. 1995).

3.5 Conclusion and Future Prospects

Species of the genus *Trichoderma* are cosmopolitan in soils and on decaying wood and herbaceous litter. *Trichoderma* species could be readily obtained by soil washing techniques on wood and can frequently be observed as discrete colonies from which isolation and pure culture can be obtained. Characteristics differentiation of different *Trichoderma* isolates can easily be observed in growing media. Culture media is a more efficient and useful tool than non-culturable methods for the isolation, quantification, and functional study of *Trichoderma* sp. Universally premiered PCR (UP-PCR) fingerprinting combined with ITS1 ribotyping is useful to differentiate the closely related strains. These techniques assembled *Trichoderma* species into 15 hereditary elements over the previous 35 years; the extent of named *Trichoderma* species has expanded from 9 total species to around 80 phylogenetic species. Some of *Trichoderma* sp. is of economic importance as they produce enzymes of industrial importance, antibiotics, and their action as biocontrol agents. This book chapter focuses on biodiversity of *Trichoderma* sp., production of secondary metabolites, various lytic enzymes by *Trichoderma* sp., and their biotechnological applications.

Furthermore, the application of fungi in combination with PGPR could be a meaningful approach for sustainable agriculture, but there are still certain aspects

which need to be further investigated so that maximum benefits could be obtained in terms of improved plant growth from this naturally occurring population mainly under stress conditions. Recently, the capability of numerous *Trichoderma* species to live as endophytes has also been recognized. Of these, *Trichoderma theobromicola*, isolated as an endophytic fungus from cacao in South America, produces a volatile/diffusible antibiotic that inhibited development of cacao frosty pod rot, *Moniliophthora roreri*, in vitro and on pod trials. These varied implications of *Trichoderma* on human society render an accurate species identification an important issue. However, due to the homoplasy of characters used, morphological determination of taxa is difficult even for experts.

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Chapter 4
Aspergillus: Biodiversity, Ecological Significances, and Industrial Applications

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Abstract Since Pier Antonio Micheli described and published genus *Aspergillus* in *Nova Plantarum Genera* in 1729, the genus attracted an immense interest. *Aspergillus*, a diverse genus occurring worldwide, species from this genus are considered to primarily be terricolous with important roles as decomposers of organic materials and cause destructive rots in the agricultural products and food industry where they produce a wide range of mycotoxins. The genus currently contains more than 340 accepted species, and its economic and historical importance makes it remain at center stage in future discussions about nomenclature and mycological diversity. Therefore, together with its ubiquitous nature, these species (anamorphic and teleomorphic) are of great significant impacts on ecosystems, agriculture, food production, biotechnology, and human and animal health. This chapter aims to give an overview on the studies and investigation of *Aspergillus* biodiversity in a wide variety of different ecological habitats, ecological significances, and industrial applications.

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4.1 Introduction

Members of the genus *Aspergillus* are cosmopolitan and prevalent components of different ecosystems in a wide range of environmental and climatic zones (Klich 2002a; Lević et al. 2013; Abdel-Azeem et al. 2016) because they can colonize a wide variety of substrates. Species belonging to the genus *Aspergillus* are widely distributed throughout the world biomes, e.g., soil (Klich 2002a; Abdel-Azeem and Ibrahim 2004; Conley et al. 2006; Jaime-Garcia and Cotty 2010), salt marshes (Abdel-Azeem 2003; Butinar et al. 2011; Balbool et al. 2013), agricultural ecosystems (Bayman et al. 2002; Horn 2003; Jaime-Garcia and Cotty 2006; Abdel-Azeem et al. 2007; Marín et al. 2012; Muthomi et al. 2012), arctic (Arenz et al. 2014), living biota (Yu et al. 2012; Salem and Abdel-Azeem 2014; Tripathi and Joshi 2015), stones (Abu Deraz et al. 2016; Tang et al. 2012), water-related (Sivakumar et al. 2006; Bonugli-Santos et al. 2015), fossils (Thomas and Poinar 1983; Dörfelt and Schmidt 2005), and human (Horré et al. 2010; Marguet et al. 2012; Findley et al. 2013; Hallen-Adams and Suhr 2017).

The occurrence of *Aspergillus* species is controlled by several factors including microclimate, the availability of substrates, as well as water activity and complex ecological interactions (Mouchacca 1995; Abdel-Azeem 2003; Grishkan and Nevo 2010; Pettersson and Leong 2011). Survival in different environmental and geographical habitats can be related to metabolic diversity, high reproductive capacity, and competitive capabilities of *Aspergillus* strains in nature (de Vries and Visser 2001; Horn and Dorner 2002; Shehu and Bello 2011; Mehl and Cotty 2013). The genus *Aspergillus* consists of about more than 340 species including both pathogenic and beneficial species (Samson et al. 2014; Abdel-Azeem et al. 2016). Several species are pathogenic to plants, animals, and humans (e.g., *A. fumigatus*, *A. terreus*) and/or produce different types of toxins, such as aflatoxins and ochratoxins (e.g., *A. flavus*, *A. ochraceus*). On the other hand, several species are widely used in different industrial applications, e.g., production of foods, drinks, organic acids, and a large variety of enzymes (e.g., *A. niger*, *A. aculeatus*, *A. oryzae*). The broad relevance and economic importance of the genus have pushed it to the forefront of fungal research, with one of the largest academic and industrial research communities dedicated to this genus.

Fungi, more specifically the aspergilli, have been highly present and necessary in this process, with their metabolites being discovered, explored, and optimized. Enzymes, organic acids, and many other molecules have brought a huge variety of products into the market and/or improved the existing ones to a level that was never before experienced. The effort of many research and industrial actors, as well as governmental policies in some cases, helped all of us to benefit from such results and evaluate the commitment and accomplishments of science. The aim of this chapter is to give an overview about the studies aimed at the investigation of *Aspergillus* biodiversity in a wide variety of different ecological habitats, ecological significances, and industrial applications.

4.2 Biodiversity of *Aspergillus* in Different Habitats

4.2.1 Desert

A “desert” is a region that receives extremely low amount of rains – less than 250 mm per year – far less than the amount required to support the growth of most elements of flora. Approximately 1/3 of Earth’s land surface is a desert with an area more than 52000 square kilometers (Abdel-Azeem et al. 2016). Studies on mycobiota of soils may be dated back to 1886 when Adametz started his pioneer study by isolation and naming 4 species of yeasts and 11 species of filamentous fungi including *Aspergillus* (Watanabe 2002). Species of *Aspergillus* are common, and they may account for up to 20% of the total species isolated in the desert (Christensen and Tuthill 1985; Abdel-Azeem and Ibrahim 2004).

Desert mycobiota of Egypt have been the target of many studies, viz., Montasir et al. (1956a, b), Mahmoud et al. (1964), Besada and Yusef (1968), Moubasher and Moustafa (1970), Moubasher and El-Dohlob (1970), Salama et al. (1971), Mouchacca (1971, 1973a, b, 1977, 1982), Naguib and Mouchacca (1970-1971), Mouchacca and Nicot (1973), Mouchacca and Joly (1974, 1976), Samson and Mouchacca (1974, 1975), Moubasher et al. (1985, 1988, 1990), Nassar (1998), Abdel-Hafez et al. (1989a, b, 1990), Abdel-Sater (1990, 2000), Abdel-Hafez and El-Maghraby (1993), Abdel-Azeem and Ibrahim (2004), Abdel-Azeem (1991, 2009), and Zohri et al. (2014). In 1970 late professor Moubasher and Moustafa (1970) surveyed the Egyptian soil fungi with special reference to *Aspergillus*, *Penicillium*, and *Penicillium*-related genera in 32 soil samples collected from the different localities in Egypt. They met 16 species of *Aspergillus*, and the highest population and occurrence were recorded for *A. niger*, *A. terreus*, *A. flavus*, and *A. sydowii*, respectively.

Mouchacca and Joly (1976) studied the biodiversity of genus *Aspergillus* in arid soils of Egypt. They collected 31 soil samples from western desert of Egypt. They collected 14 soils from regions receiving very weak to null winter rains and 17 samples from regions that benefit from an appreciable amount of wintry precipitation. They showed that the taxonomic distribution is hardly affected by the dimensions of soil sand components, while regional localization exerts a certain influence. They recovered 27 species of *Aspergillus*; some are practically omnipresent (*A. niger*, *A. flavus* group), and others develop preferentially in very weak to null winter soil (*A. nidulans*, *A. ustus*, *A. ochraceus*, and possibly *A. fumigatus* groups) and/or have distribution positively affected (*A. flavipes* and *A. terreus*) or perhaps negatively (*A. fumigatus* group) due to soil reclamation.

In his extensive survey of ascospore-producing taxa in Egypt, Abdel-Azeem (2003) recorded 12 anamorphic *Aspergillus* species and 7 teleomorphic forms from all habitats investigated by him including desert soil, salt marshes, cultivated soil, stored seeds and grains, air, and coprophilous dung. In their extensive survey of Sinai terricolous fungi, Abdel-Azeem and Ibrahim (2004) and Abdel-Azeem (2009)

recorded 17 species of *Aspergillus*. They recorded *A. alutaceus*, *A. candidus*, *A. clavatus*, *A. flavus*, *A. fumigatus*, *A. japonicus*, *A. niger*, *A. ochraceus*, *A. sydowii*, *A. tamarii*, *A. terreus*, *A. ustus*, *A. versicolor*, *A. wentii*, *Emericella nidulans*, *Eurotium amstelodami*, and *E. chevalieri*.

Six taxa are introduced, as new to the science, to the genus *Aspergillus* as novel taxa based on type materials collected from Egyptian deserts, namely, *Aspergillus egyptiacus* Moub. & Moustafa [as *Aspergillus aegyptiacus*] (1972), *A. floriformis* Samson & Mouch (1975), *A. pseudodeflectus* Samson & Mouch. (1975), *Emericella desertorum* Samson & Mouch (Samson & Mouchacca 1974), *E. purpurea* Samson & Mouch. (1975), and *Eurotium xerophilum* Samson & Mouch. (1975). In Libya, few investigations have been made on soil mycobiota. Naim (1967a, b) studied rhizosphere and soil fungi of *Artemisia herba-alba* and fungi under *citrus* trees in Tripoli. Youssef (1974) studied the fungal biota of Libyan soil and examined 16 different localities for their fungal microbiota. El-Said and Saleem (2008) studied soil fungi at western region of Libya. In 2010, Mansour studied the distribution and occurrence of various groups of fungi in different kinds of soils in eastern region of Libya. Result showed that the most abundant species were *Aspergillus flavus*, *A. fumigatus*, *A. niger*, *A. ochraceus*, *A. terreus*, and *A. ustus*. For more details concerning the checklist of Libya fungi, please check El-Buni and Rattan (1981).

Mycobiota of Algerian, Tunisian, and Moroccan deserts do not receive that much attention from mycologists, and hence few studies are already published concerning the mycobiota of these deserts. Recently mycobiota *of* three chotts located in the northeast of Algerian Sahara have been studied by *Dendouga* et al. (2015). Authors isolated 327 colonies of fungi, and *Aspergillus* was one of the most common genera isolated in this study (Abdel-Azeem et al. 2016). Various studies carried by several investigators on micromycetes of the Kingdom of Saudi Arabia desert soils showed that *A. amstelodami*, *A. chevalieri*, *A. ruber*, *A. ochraceus*, *A. fumigatus*, *A. flavus*, *A. sydowii*, *A. terreus*, and *A. ustus* are the most common species (Fathi et al. 1975; Ali 1977; Ali et al. 1977; Abdel-Hafez 1982a, b, c, 1994; Hashem 1991, 1995; Arif and Hashem 1988; Barakat 1999; Saadabi 2006; Abou-Zeid and Abd El-Fattah 2007). Also the teleomorph genera *Emericella* (*E. nidulans*) and *Eurotium* with *E. amstelodami* and *E. chevalieri* are common in Saudi Arabia desert soils (Abdel-Azeem et al. 2016).

Tolba et al. (1957), *Al-Doory* et al. (1959), Ismail and Abdullah (1977), and Abdullah et al. (1986) studied soil microfungi from different localities in Iraq. In these studies, the genus *Aspergillus* accounted for about 16% of the total species isolated. *A. fumigatus* was the most common species, being isolated from 70% of the sampling sites examined. *Aspergillus candidus* and *A. niger* came in the second and third position in frequency, being isolated from 60 and 50% of the sampling sites examined, respectively. Imran and Rubaay (2015) studied the molecular ecological typing of environmental isolates of *A. terreus* that were collected from the desert region in Iraq. In Syria various species of *Aspergillus* were recorded by various investigators like Sizova and Gorlenko (1967), Baghdadi (1968), Abdel-Hafez et al. (1983), and Abdel-Kader et al. (1983). *Aspergillus niger*, *A. sydowii*, *A. flavus*, *A. wentii*, and *A. clavatus* were the most prevalent species. *Aspergillus kassunensis*

as a new species added to genus *Aspergillus* was introduced by Baghdadi (1968) from Syrian soil.

Al-Subai (1983) and Moubasher (1993) concluded that *Aspergillus* was consistently the most common genus in Qatari soils. Moubasher (1993) isolated fungi from 11 desert soil samples out of 42 samples representing different ecological habitats of Qatar. *Aspergillus* contributed by 23 species and 5 varieties of which *A. terreus*, *A. flavus*, *A. versicolor*, and *A. niger* were the most frequent species. Halwagy et al. (1982) found *Aspergillus*, *Alternaria*, and *Drechslera* constituted 16%, 5%, and 3% respectively of the total species isolated from desert soils in Kuwait. They recorded *Aspergillus terreus, A. fumigatus*, and *A. niger* with frequencies of occurrence of 70%. In 1994 El-Said studied soil mycoflora of Bahreen (Bahrain) in which 39 species belonging to 20 genera were isolated from 50 soil samples on different isolation media. *Aspergillus flavus*, *A. fumigates*, *A. niger*, *A. sydowii*, and *A. terreus*, *Eurotium amstelodami*, and *E. chevalieri* were the most common species.

Mycobiota of the northern part of the Negev desert (Rayss and Borut 1958; Borut 1960; Guiraud et al. 1995; Steiman et al. 1995) are represented by 159 species belonging to 58 genera, in which 16 of them are under the genus *Aspergillus*. *Aspergillus fumigatus*, *A. sclerotiorum*, and *A. versicolor* are the most common species in this region. Volz et al. (2001) concluded that the majority of Israel (occupied by Palestine) soil fungi (309 species – 70%) belong to the division *Ascomycota*, but only 56 species of them were found to have a perfect stage in their life cycle. Concerning species diversity among genera, they showed that *Aspergillus* recorded only 48 species (15.53%) out of 309 species. *Aspergillus niger*, *A. terreus*, *A. ustus*, and *A. versicolor* are the most widely distributed species in Israel. Grishkan and Nevo (2010) isolated 185 species belonging to 76 genera from the soil of Makhtesh Ramon hot desert in Israel (occupied Palestine). Ten species of *Aspergillus*, nine anamorphic and one teleomorphic, were isolated, in which *A. fumigatus* comprised a basic part of thermotolerant mycobiota obtained in this study.

Aspergillus as xerotolerant and xerophilic genus can grow at or below a water activity (a_w) of 0 (Pettersson and Leong 2011). Several researchers have isolated genus *Aspergillus* from desert soils in Argentina, Chile, and Mexico (Giusiano et al. 2002, Piontelli et al. 2002, Samaniego-Gaxiola and Chew-Madinaveitia 2007). Conley et al. (2006) studied the fungal content of Atacama Desert, the driest and oldest desert on Earth, without any recorded rainfall for decades. They reported 12 genera of fungi, *Aspergillus* one of them. *Aspergillus flavus* and *A. fumigatus* reported from desert soils worldwide (Moubasher 1993; Abdel-Hafez 1981; Giusiano et al. 2002; Abdel-Azeem 2003; Piontelli et al. 2002; El-Said and Saleem 2008) and *A. carneus* recorded exclusively from desert soils in the Middle East (Abdullah et al. 1986; Ali-Shtayeh and Jamous 2000; El-Said and Saleem 2008) were missing in the Atacama soil. Grishkan et al. (2015) examined the variations in microfungal communities inhabiting different biological crust types in the vicinity of the Shapotou Research Station in the Tengger Desert, China. The mycobiota isolated from the crusts sampled in 2011 and 2013 was composed of 123 and 67 identified species, respectively. She and her team recovered 134 species: 6 of

Mucoromycotina, 22 of teleomorphic (morphologically sexual) *Ascomycota*, and 106 of anamorphic (asexual) *Ascomycota*. These species belonged to 66 genera, with the most common being *Aspergillus* (12 species). Taxa of *Aspergillus fumigatus*, *A. niger*, *A. nidulans*, and *A. rugulosus* were dominated.

Klich (2002a) published her biogeography of *Aspergillus* species in soil and litter, and she concluded that there was no overall trend in the distribution of the members of the entire genus by ecosystem; however, individual sections of the genus appeared to have distinct distribution patterns. Most members of sections *Aspergillus*, *Nidulantes*, *Flavipedes*, and *Circumdati* occurred at greater than expected frequencies in desert soils (Klich 2002a). To conclude, in deserts environments, the pan-global stable *Aspergillus* species are represented by *A. niger*, *A. flavus*, *A. fumigatus*, *A. ochraceus*, *A. terreus*, *A. sydowii*, *A. tamarii*, *A. ustus*, *A. versicolor*, *A. wentii*, *Emericella nidulans*, *Eurotium amstelodami*, and *E. chevalieri* (Abdel-Azeem et al. 2016).

4.2.2 Salt Marshes

When evaporation of seawater is accompanied with halite (NaCl) concentrations greater than 10% (m/w), thalassohaline hypersaline environments originated (Oren 2002) and provide some of the most extreme habitats in the world. They are common all around the globe and include, for example, marine ponds and salt marshes that are subjected to evaporation, salt or soda lakes, and sea-salt and man-made salterns (Trüper and Galinski 1986). Life-limiting parameters in salterns are many, e.g., variable water activities (a_w), high concentrations of NaCl, low oxygen concentrations, as well as high light intensity (Brock 1979). Halotolerant and halophilic fungi were first reported as active inhabitants of solar salterns by Gunde-Cimerman et al. (2000). Later on, they were isolated by several investigators (Butinar et al. 2005a, b, c; Cantrell et al. 2006) from salterns around the world, e.g., La Trinidad in the Ebro River Delta and Santa Pola on the Mediterranean coast of Spain, Camargue in France, and the salterns on the Atlantic coast in Portugal and in Namibia, the Dominican Republic, and Puerto Rico. After a decade of research into the fungal diversity in salterns, together with new taxa, a number of fungal genera with high diversities of halotolerant and halophilic species have been described. Different species of genus *Aspergillus* are among the filamentous fungi that appear with the highest frequencies in salterns (Butinar et al. 2011). The group of filamentous fungi that have been isolated from different salterns around the world is mainly represented by the order *Eurotiales* by the teleomorphic genera *Eurotium* and *Emericella* and the anamorphic *Aspergillus* and *Penicillium* (Tresner and Hayes 1971; Abdel-Azeem 2003; Cantrell et al. 2006; Butinar et al. 2011).

Butinar et al. (2011) listed *Aspergillus melleus*, *A. sclerotiorum*, and *Petromyces alliaceus* (holomorphic species) within these taxonomic groups, although they have appeared only locally. Both *Aspergillus versicolor* and *A. sydowii* have also been identified as part of the fungal communities in the hypersaline environments even if

they are common in marine environments and in dry foods. *Aspergillus wentii*, *A. flavipes*, *A. terreus*, and particularly *A. candidus* have been repeatedly isolated from Adriatic salterns, whereas *A. penicillioides*, *A. proliferans*, and *A. restrictus* have been found only sporadically at salinities below 10% NaCl. *Aspergillus fumigates* is common in arid environments (deserts) at high temperatures and has been found consistently in solar salterns, although it is also most abundant at salinities below 10% NaCl (Moustafa 1975; El-Dohlob and Migahed 1985; Moubasher et al. 1990; Abdel-Azeem 2003; Abdullah et al. 2010; Butinar et al. 2011; Balbool et al. 2013). Six different species of the known teleomorphic foodborne xerophilic genus *Eurotium* were repeatedly isolated in a mycodiversity study of hypersaline waters, *Eurotium amstelodami*, *E. herbariorum*, and *E. repens* as indigenous taxa in hypersaline water, while *E. rubrum*, *E. chevalieri*, and *E. halotolerans* are only impermanent inhabitants of brine at lower salinities (Butinar et al. 2005c). To conclude, in hypersaline environments, the pan-global stable taxa of genus *Aspergillus* are represented by *A. niger* and *E. amstelodami* and possibly also by *A. sydowii*, *A. candidus*, and *E. herbariorum*, which are also quite abundant, although more locally distributed (Butinar et al. 2011).

4.2.3 Polar

Around 2.3% of the world's fungal biota exists in the Arctic, and fungi in this region have been isolated from various substrates and habitats (Ivarson 1965; Reeve et al. 2002; Säwström et al. 2002; Callaghan et al. 2004; Ozerskaya et al. 2009; Pathan et al. 2009). More than 1000 species and over 400 genera of non-lichenized fungi are reported from Antarctic regions (including the sub-Antarctic) (Bridge and Spooner 2012; Arenz et al. 2014) including genus *Aspergillus*. The genus *Aspergillus* is also mesophilic to thermotolerant, yet some spores of *Aspergillus* and its associated teleomorphs are found in Arctic regions (Gunde-Cimerman, et al. 2005). The presence of "cosmopolitan" species such as *Alternaria*, *Penicillium*, *Aspergillus*, *Cladosporium*, and others may be refereed to their wide dispersal potential and ubiquitous association with human activities and material (Ruisi et al. 2007).

However, fungal diversity in Arctic soils has been investigated only to a limited extent. Krishnan et al. (2011) isolated 28 isolates of fungi from bird-forming soil and pristine and human-impacted soils collected from the Fildes Peninsula, King George Island, Antarctica, without any *Aspergillus* species. Singh et al. (2012a, b) studied filamentous soil fungi from Ny-Ålesund, Spitsbergen, and they isolated 19 species under 14 genera. *Aspergillus* is represented by three species, namely, *Aspergillus aculeatus*, *A. flavus*, and *A. niger*. Similarly, other genera seem to be absent in cold ecosystems, for example, *Byssochlamys* and its anamorphic state *Paecilomyces*. *Aspergillus* species in general grow poorly below 12 °C and thus may have been recovered as spores in cold ecosystems (Gunde-Cimerman et al. 2003) because they are common as marine spores, are transported by wind or birds, or are carried around due to human activity (Frisvad 2008).

4.2.4 Agricultural

Globally the majority of the research which involved the isolation and identification of *Aspergillus* strains from various agricultural and horticultural crop fields in different agroclimatic zones was undertaken in order to evaluate them for mycotoxin production (Klich 2002b). Therefore, only a limited number of studies deal with biodiversity of the genus *Aspergillus* in specific crop fields or agroecosystems. Climatic factors, followed by edaphic and spatial patterning, are the best predictors of soil fungal richness and community composition at the global scale (Tedersoo et al. 2014). Biotic (plant species and their growth stage, microbial competition) and abiotic factors (soil physicochemical characters, application of pesticides and/or fertilizers) as well as the geographical position affected populations and diversity of fungal communities in agroecosystems (Kredics et al. 2014). In her biogeographic study of *Aspergillus* species in soil and litter, Klich (2002a) found that five species of *Aspergillus* reported in over 100 studies were *A. fumigatus*, *A. versicolor*, *A. terreus*, *A. flavus*, and *A. niger* var. *niger*. With one exception, these five species occurred at the expected frequencies in all of the biomes; *A. terreus* occurred at greater than expected frequencies in cultivated soils and less than expected frequencies in forest soils. In many parts of Egypt, several investigators studied soil fungi from cultivated soil, e.g., Abdel-Hafez (1974), Moubasher and Abdel-Hafez (1978), and Abdel-Azeem (2003). They found taxa belonging to *Aspergillus*, *Penicillium*, *Fusarium*, and *Mucor*, and some dematiaceous hyphomycetes were the most common in various types of Egyptian soils. In 1983 Mazen and Shaban studied the fluctuation of soil fungi in wheat fields and found that the most common fungi isolated were *Aspergillus* represented by five species *Aspergillus niger*, *A. terreus*, *A. fumigatus*, *A. flavus*, and *A. versicolor.* Abdel-Hafez and his coworkers (2000) isolated 118 species in addition to 7 varieties belonging to 51 genera from cultivated and desert soils in Egypt. The results obtained from the three soil types were basically similar, and the most common *Aspergillus* species were *A. flavus*, *A. flavus* var. *columnaris*, *A. fumigatus*, *A. niger*, *Aspergillus sydowii*, and *A. terreus*.

Hafez (2012) made an ecological comparison on soil and rhizospheric fungi of maize and wheat plants in different areas in Minya Governorate in Egypt. She isolated 28 fungal species from wheat belonging to 18 genera and that 13 species were isolated from maize belonging to 9 genera. *Aspergillus* was the most dominant in both rhizospheric and non-rhizospheric soils and represented by four species; they were *A. niger*, *A. terreus*, *A. flavus*, and *A. ustus*.

Fusaria and other fungi associated with rhizosphere and rhizoplane of lentil and sesame at different growth stages from cultivated soil in Egypt have been studied by Abdel-Hafez et al. (2012). They isolated 16 *Fusarium* species, and 3 *Aspergillus* species (*Aspergillus flavus*, *A. niger*, and *A. ochraceus*) were isolated. Abdel-Azeem et al. (2007) studied the effects of long-term heavy metal contamination on diversity of terricolous fungi and nematodes in agroecosystem in Egypt as a case study. They collected 100 soil samples in a randomized way to represent different stages of land reclamation during the period from September (2004) to February (2005).

These profiles represented different land use periods of 0–20 years. Isolated species belonged to 21 genera. The prevailing genera were *Aspergillus* (12 species including anamorph stages of one *Emericella* and one *Eurotium* species; 52.63% of the total isolates). They found that the most abundant species were *Aspergillus niger* var. *niger* (21.15% of the total isolate number), *Trichoderma pseudokoningii* (12.65%), *A. flavus* (9.4%), and *A. fumigatus* (8.63%).

Aspergillus taxa distributed in different altitudes (24 m above sea level to 2000 m above sea level) of Eastern Himalayas were studied by Devi and Joshi (2012). They recorded *Aspergillus versicolor* in samples collected from 1–500 m above sea level, *Aspergillus nomius* 500–1000, *Aspergillus niger* 1000–1500, and *Aspergillus fumigates*, *A. flavus*, *A. terreus*, and *A. awamori* 1500–2000. *Aspergillus* species are able to produce a range of mycotoxins, including aflatoxins, ochratoxins, fumonisins, and patulin. Aflatoxins are mainly produced by members of *Aspergillus* section *Flavi*, and they contaminate various agricultural products in several parts of the world (Baranyi et al. 2013).

Taxonomically, based on *Aspergillus* species, mycotoxins in fruits can be divided into three major groups: (1) aflatoxins produced by *A. flavus*, *A. parasiticus*, and *A. nomius*; (2) ochratoxin A produced by *A. ochraceus*, *A. carbonarius*, *A. niger* aggregate, *A. tubingensis*, *A. sclerotiorum*, *A. sulphureus*, *A. aculeatus*, *A. japonicus* var. *aculeatus*, *A. alliaceus*, *A. melleus*, and other species; and (3) other toxic metabolites produced by a variety of *Aspergillus* spp., the most important of these being sterigmatocystin, produced by *A. flavus*, *A. flavipes*, *A. nidulans*, and *A. versicolor*; cyclopiazonic acid, produced by *A. flavus*, *A. tamarii*, and *A. versicolor*; aflatrem, produced by *A. flavus*; citrinin, produced by *A. flavipes*, *A. carneus*, *A. niveus*, and *A. terreus*; and patulin, produced by *A. terreus* (Gill-Carey 1949; Raper and Fennell 1965; Semeniuk et al. 1971; Ciegler 1972; Hesseltine et al. 1972; Buchanan et al. 1975; Durley et al. 1975; Lee et al. 1975; Mislivec et al. 1975; Sommer et al. 1976; Moss 1977; Gallagher et al. 1978; Stack and Mislivec 1978; Gorst-Allman and Steyn 1979; Anke et al. 1980; Cole and Cox 1981; Davis 1981; Wicklow and Cole 1982; Turnerr and Aldridge 1983; Cole 1984; Dorner et al. 1984; Scudamore et al. 1986; Kurtzman et al. 1987; Vesonder et al. 1988; Betina 1989; Kim et al. 1993; Doster et al. 1996; Varga et al. 1996; Richard et al. 1999; Giridhar and Reddy 2001; Sage et al. 2002, 2004; Battilani and Pietri 2002; Bayman et al. 2002; Serra et al. 2003; Magnoli et al. 2004; Iamanaka et al. 2005; Medina et al. 2005; Perrone et al. 2006; Roussos et al. 2006; Barkai-Golan and Paster 2008).

Fourteen species assigned to three sections of the genus *Aspergillus* are responsible for acute aflatoxicosis epidemics that occurred recently in several parts of Asia and Africa leading to death of several hundred people. Taxa were distributed among three sections: section *Flavi* (*A. flavus*, *A. pseudotamarii*, *A. parasiticus*, *A. nomius*, *A. bombycis*, *A. parvisclerotigenus*, *A. minisclerotigenes*, *A. arachidicola*, *A. togoensis*), section *Nidulantes* (*Emericella astellata*, *E. venezuelensis*, *E. olivicola*), and section *Ochraceorosei* (*A. ochraceoroseus*, *A. rambellii*) (Varga et al. 2009; Rank et al. 2011). Potential aflatoxin-producing *A. flavus* isolates were also identified in other agricultural products including stored wheat, onions, grapes, and rice and in cattle feed (Krnjaja et al. 2008). Aflatoxins were also detected in sunflower

flour samples (Masic et al. 2003) and in spices in Serbia (Saric and Skrinjar 2008). Several *Aspergillus* species are also able to produce patulin, including species assigned to *Aspergillus* sections *Clavati* (Varga et al. 2007b) and *Terrei* (Varga et al. 2005). These species frequently occur in cereals and cereal products (Lopez-Diaz and Flannigan 1997; Abramson et al. 1987). The most well-known species *A. clavatus* can be isolated mainly from soil and dung, but it also occurs in stored products (mainly cereals) with high moisture content, e.g., inadequately stored rice, corn, and millet (Flannigan and Pearce 1994). *A. clavatus* isolates appeared to be particularly well adapted for growth during malting (Flannigan and Pearce 1994).

4.2.5 Water-Related

Shearer et al. (2007) estimated fungal biodiversity in freshwater, brackish, and marine habitats based on reports in the literature. In their study they covered the ecological group which includes fungi and taxa formerly treated as fungi, exclusive of yeasts, in freshwater, brackish, and marine habitats. They reported approximately 3047 taxa from aquatic habitats thus far. The largest taxonomic group of fungi in aquatic habitats is comprised of teleomorphic and anamorphic *Ascomycota*, followed by the *Chytridiomycota*. *Marine fungi are an ecological* rather than a taxonomic *group* and comprise an estimated 1500 species, excluding those that form lichens (Hyde et al. 1998). Obligate marine fungi grow and sporulate exclusively in the marine or estuarine environment; facultative marine species may grow in marine as well as in freshwater (or terrestrial) habitats (Kohlmeyer and Kohlmeyer 1979). A case in point is *Aspergillus sydowii*, isolated from diseased sea fans and causing the disease in laboratory experiments (Geiser et al. 1998). *In 1997 Boutaiba studied* fungal flora of Lake El Golea in Algeria. He studied their taxonomy, ecology, and metabolite production. *He isolated Aspergillus niger*, *A. terreus*, *A. sydowii*, *A. repens*, *A. ochraceus*, *A. fumigatus*, *A. flavus*, *A. candidus*, and *A. wentii.*

Singh et al. (2012a, b) investigated fungal diversity in two sediment cores w40 cmbsf (cm below seafloor) at a depth of w5 000 m in the Central Indian Basin (CIB), by culture-dependent as well as culture-independent approaches. This resulted in recovering a total of 19 culturable fungi and 46 operational taxonomic units (OTUs), respectively. The majority of the fungi belonged to *Ascomycota*, within no single species dominating. It included members of filamentous fungi such as *Aspergillus* sp., *Eurotium* sp., *Cladosporium* sp., *Pleospora* sp., *Chaetomium* sp., *Ascotricha* sp., *Penicillium* sp., and *Sagenomella* sp.

Zhang et al. (2014) investigated the composition and abundance of fungal community in the deep-sea sediments of the Pacific Ocean. They identified 12 ascomycetes that belonged to six genera (*Aspergillus*, *Aureobasidium*, *Candida*, *Exophiala*, *Fusarium*, and *Periconia*). *Aspergillus* is represented only by two species *Aspergillus sydowii* and *A. vitricola*. Abdel-Azeem et al. (2015) studied the occurrence and diversity of mycobiota in heavy metal-contaminated sediments of Mediterranean coastal lagoon El-Manzala, Egypt. They found that the prevailing genera were

Aspergillus (11 species including anamorph stages of two *Emericella* species; 36.66% of the total isolates) and *Penicillium* (4 species including anamorph of *Talaromyces*; 13.33%), and the remaining taxa were represented only by two to one species each. *Aspergillus niger*, *A. flavus*, and *A. terreus* showed the highest percentage of frequency of occurrence (Abdel-Azeem et al. 2015).

4.2.6 Mangrove

Mangroves are an assortment of tropical and subtropical trees and shrubs which have adapted to the inhospitable zone between sea and land: the typical mangrove habitat is a muddy river estuary (Kathiresan and Bingham 2001; Hogarth 2007). Mangles are considered a dynamic ecotone, and approximately 25% of the world's coastline is dominated by mangroves distributed in 112 countries encompassing an area of 18 000000 ha (Spalding et al. 1997). Biodiversity of biota associated with mangle ecosystem is well known for animals and plants but poorly known for fungi (Khalil et al. 2013). Species diversity of fungi, seasonal variation, and frequency of occurrence in Muthupettai mangroves, east coast of Tamil Nadu, India, were studied at two different seasons by Sivakumar et al. (2006). A total number of 118 fungal species were isolated, of which maximum 94 species from sediment samples followed by water with 83 species in which *Aspergillus* came first as the common genus followed by *Penicillium*, *Curvularia*, and *Alternaria*.

Tariq et al. (2008) studied the rhizosphere fungi of four different species of mangrove plants collected from coastal areas in Pakistan. They found that *A. flavus*, *A. fumigatus*, and *A. niger* were common in the rhizosphere soil of the four species of mangrove plants sampled. Behera et al. (2012) studied the diversity of soil fungi from mangroves of Mahanadi delta, Orissa, India. Twenty-two fungal species and *A. oryzae*, *A. niger*, *A. flavus*, and *A. albus* as occasionally frequent were recorded. Madavasamy and Pannerselvam (2012) studied the phylloplane fungi of green, senescent, and brown leaves of *Avicennia marina*. Recovered taxa included *Aspergillus candidus*, *A. flavus*, *A. luchuensis*, *A. niger*, *A. sydowii*, *A. fumigatus*, and *A. sulphureus* out of total of 22 species. The mycobiota composition of the mangrove soil located in coastal area at Red Sea in Egypt was investigated in 24 soil samples that were collected (Khalil et al. 2013). *Aspergillus flavus*, *A. niger*, *A. versicolor*, and *A. fumigatus* were recorded with high species frequency in more than 15 cases out of 24.

4.2.7 Living Plants, Lichens, and Animals

Endophytes colonize symptomlessly the living, internal tissues of their host, even though the endophyte may, after an incubation or latency period, cause disease (Petrini 1991). In literature the term "fungal endophytes" is normally used to

describe fungal organisms, which, in contrast to rhizal fungi, reside entirely within the host tissues and emerge during host senescence (Rodriguez and Redman 2008). Endophytic fungi have been classified into two groups based on differences in taxonomy, evolution, plant hosts, and ecological functions: clavicipitaceous, which are able to infect only some species of grasses, and non-clavicipitaceous, which are found in the asymptomatic tissues of bryophytes, ferns, gymnosperms, and angiosperms (Rodriguez et al. 2009). There are 1.3 million species of endophytic fungi alone, the majority of which are likely found in tropical ecosystems (Verma et al. 2014). There has been great interest in endophytic fungi as potential producers of novel biologically active products (Yadav 2018; Schulz et al. 2002; Wildman 2003; Strobel and Daisy 2003; Tomita 2003; Urairuj et al. 2003; Spiering et al. 2006; Manoharachary et al. 2013; Suman et al. 2016; Yadav et al. 2018).

Unique species of endophytic fungi with a wide range of potential practical applications in plant protection as repellents, insecticides, antimicrobials, anthelmintic, and vermicides have been found (Rana et al. 2017; Rana et al. 2016a; Rana et al. 2016b; Strobel et al. 2008; Vega et al. 2008). In the last 5 years, there is evidence of the use of endophytes for producing anticancer, antimicrobial, and antioxidant compounds and also in biotransformation process (Pimentel et al. 2011; Salem and Abdel-Azeem 2014). Species of *Aspergillus* as a member of non-clavicipitaceous endophytes attracted the attention of researchers as effective producers of bioactive metabolites. Such studies may result in the description of new *Aspergillus* species, e.g., Zhao et al. (2009) described *Aspergillus niger* var. *taxi* as a new species variant of taxol-producing fungus isolated from *Taxus cuspidata* in China. Endophytic fungi *Aspergillus clavatus* isolated from *Azadirachta indica* plant have also been reported to synthesize silver nanoparticles which have significant antibacterial and antifungal activity (Verma et al. 2010). Endophytic *Aspergillus fumigatus* isolated from *Juniperus communis* as a novel source of the anticancer prodrug deoxypodophyllotoxin has been isolated and chemically characterized by Kusari et al. (2009).

Mustafa et al. (2013) exploited some Egyptian endophytic taxa for extracellular biosynthesis of silver nanoparticles. They isolated endophytic fungi from medicinal plants in arid Sinai. Their results showed that *Zygomycota* is represented by two species (9.5% of the total species number): teleomorphic *Ascomycota* (3 species, 14.2%) and anamorphic *Ascomycota* (16 species, 76.19%). The prevailing genera were *Aspergillus* (3 species including anamorph stages of one *Eurotium* species; 14.28% of the total isolates) and *Alternaria* (2 species, 9.5%). The remaining taxa were represented only by one species each. The most abundant species were *Alternaria alternata* (41.6%), *Nigrospora oryzae* (38.3%), and *Chaetomium globosum* (11.1%). A total 13 species belonging to 11 genera were screened for the production of AgNPs. They recorded that *Aspergillus niger* synthesized AgNPs in a moderate rate in comparison with other taxa.

Silva et al. (2011) studied endophytic fungi from *Laguncularia racemosa* (Brazilian mangrove) and their antimicrobial potential. They recovered 6 isolates of *Aspergillus niger* out of 70 endophytic strains. Zhang et al. (2012a, b) isolated indolyl diketopiperazines (**1–6**) from the endophytic fungus *Aspergillus tamarii* of

Ficus carica and examined its anti-phytopathogenic potentiality in vitro for the first time. Thirty-nine fungal metabolites, including two new alkaloids, of endophytic fungus *Aspergillus fumigatus* isolated from the stem bark of *Melia azedarach* and their antifungal, antifeedant, and toxic activities were tested by Li et al. (2012). Palencia (2012) studied endophytic associations of species in the *Aspergillus* section *Nigri* with *Zea mays* and *Arachis hypogea* and their mycotoxins. He developed a system to identify black aspergilli from peanut and maize in the Southeastern United States. His survey indicated that *A. niger* species complex is predominant in maize and peanut fields. Raghunath et al. (2012) screened *Aspergillus niger* isolated from *Taxus baccata* for the production of lovastatin on a solid-state fermentation. The presence of lovastatin was confirmed by different techniques, e.g., spectroscopic method, nuclear magnetic resonance (NMR), thin-layer chromatography (TLC), and high-performance liquid chromatography (HPLC) methods.

Guatam (2014) isolated endophytic fungi from leaf segments of five medicinal plants collected from Mandi District, Himachal Pradesh, India. *Aspergillus niger*, *A. flavus*, *A. clavatus*, and *A. variecolor* were isolated with 14 species belonging to 15 genera of total 373 fungal strains. Eight medicinal plants (*Achillea fragrantissima*, *Artemisia herba-alba*, *Chiliadenus montanus*, *Origanum syriacum*, *Phlomis aurea*, *Tanacetum sinaicum*, *Teucrium polium*, and *Thymus decussates*) were screened for their content of endophytic fungi on different altitudes by Salem and Abdel-Azeem (2014) in Saint Katherine Protectorate, South Sinai, Egypt. Salem and Abdel-Azeem isolated 32 genera belonging to 75 species in which 9 species of *Aspergillus*, namely, *A. alliaceus*, *A. bisporus*, *A. candidus*, *A. flavus*, *A. fumigatus*, *A. japonicus*, *A. niger*, *A. terreus*, and *A. versicolor*, were recovered. Yu et al. (2012) studied the diversity of endozoic fungi in the South China Sea sponges and their potential in synthesizing bioactive natural products suggested by PKS gene and cytotoxic activity analysis. They isolated 14 genera, and *Aspergillus* came first as the predominant component in the culturable fungal community and was represented by *Aspergillus insulicola*, *A. penicillioides*, *A. terreus*, *A. oryzae*, and *E. rubrum*. Genus *Aspergillus* is associated with more than 30 species of sponge all over the world (Abrell et al. 1996; Varoglu and Crews 2000; Lin et al. 2003; Gao et al. 2008; Proksch et al. 2008; Ein-Gil et al. 2009; Li and Wang 2009; Lee et al. 2010; Liu et al. 2010; Menezes et al. 2010; Paz et al. 2010; Ding et al. 2011; Wiese et al. 2011; Zhou et al. 2011; Thirunavukkarasu et al. 2012; Yu et al. 2012). The most common species of *Aspergillus* recorded in those studies were *A. aculeatus*, *A. insuetus*, *A. niger*, *A. ostianu*, *A. sclerotiorum*, *A. ustus*, *A. versicolor*, and *Eurotium cristatum* (Suryanarayanan 2012).

Bai et al. (2014) characterized two new aromatic butyrolactones, flavipesins A (**1**) and B (**2**), two new natural products (**3** and **4**), and a known phenyl dioxolanone (**5**) from marine-derived endophytic fungus *Aspergillus flavipes*. Different species from the genus *Aspergillus* are cited as marine-derived producers of enzymes (Bonugli-Santos et al. 2015). *Aspergillus terreus* was most frequently isolated as an endosymbiont from green, brown, and red seaweeds, namely, *Caulerpa scalpelliformis*, *Halimeda macroloba*, *Ulva lactuca*, *U. fasciata*, brown *Lobophora variegata*, *Padina gymnospora*, *Stoechospermum marginatum*, *Sargassum ilicifolium*,

Portieria hornemanni, and *Gracilaria edulis*, respectively (Suryanarayanan et al. 2010). Marine-derived fungi such as *Aspergillus* spp., apart from dominating the endosymbiont assemblage of seaweeds (Suryanarayanan et al. 2010), dominate the fungal consortium of marine invertebrates collected from different localities such as the Great Barrier Reef, North Sea, the Mediterranean, and the Caribbean (Höller et al. 2000), referring to their adaptation to occupy such microniches. Such a widespread occurrence of marine-derived fungi may be indicative of their passive migration from terrestrial habitats (Alva et al. 2002).

Marine-derived fungi such as *Aspergillus* spp., apart from dominating the endosymbiont assemblage of seaweeds (Suryanarayanan et al. 2010), also dominate the fungal assemblages of marine invertebrates of different geographical locations (Höller et al. 2000), attesting to their adaptation to occupy such a niche as the inner tissues of seaweeds or marine animals. Such a widespread occurrence of marine-derived fungi may be indicative of their passive migration from terrestrial habitats (Alva et al. 2002). However, since these fungi are better adapted to marine environments than their terrestrial conspecifics (Zuccaro et al. 2004; König et al. 2006) and survive in seaweeds which produce antifungal metabolites (Kubanek et al. 2003; Lam et al. 2008), it is likely that they are not casual residents of the seas but have coevolved with the seaweeds (Zuccaro et al. 2004; Suryanarayanan et al. 2010). Common endosymbiont of algae and seaweeds are *Aspergillus versicolor*, *A. terreus*, *A. niger*, *A. flavus*, and *A. oryzae* (Suryanarayananr 2012).

Kelecom (2002) predicted a relationship between the type of secondary metabolite and the source of fungus, rather than the fungi themselves. The latter was exemplified by the fungi in the genus *Aspergillus* that produce fumiquinazoline derivatives if they are obtained from fish, sesquiterpene nitrobenzoate derivatives if they originate from algae, and indole diketopiperazine derivatives if they are isolated from sponges.

To conclude, in association with seaweeds, the marine-derived *Aspergillus* species are represented by *Aspergillus versicolor*, *A. terreus*, *A. niger*, *A. flavus*, and *A. oryzae* (Belofsky et al. 1998; Lee et al. 2003; Zhang et al. 2007a, b, c; Lin et al. 2008; Qiao et al. 2010). Endolichenic fungi represent an important ecological group of species that form associations with lichens, and to extend the knowledge of their diversity within macrolichens, Tripathi and Joshi (2015) isolated and identified the endolichenic fungi from some healthy macrolichens of Kumaun Himalaya. The majority of endolichenic fungi belonged to anamorphic *Ascomycota* (hyphomycetes), and the lowest were obtained from zygomycetes. *Aspergillus flavus* and *A. niger* were common as endolichenic species and recorded during various studies (Suryanarayanan et al. 2005; Li et al. 2007; Tripathi et al. 2014a, b, c; Tripathi and Joshi 2015).

4.2.8 Air and Settled Dust

Over 225 species of fungi have been reported from indoor environments which represent a few of the proposed estimate, 1.5 million species, of fungi (McGinnis 2007). The most common allergenic fungal genera are *Cladosporium*, *Alternaria*,

Aspergillus, and *Fusarium* where more than 80 genera of fungi have been linked with symptoms of respiratory tract allergies (Horner et al. 1995). Exposure to the large concentration of conidia of the four genera is considered the main causative agent of aspergillosis (Anderson et al. 1996), asthma and pneumonitis (Cuijpers et al. 1995; Hu et al. 1997), and allergic alveolitis and toxicosis (Flannigan et al. 1991). Fröhlich-Nowoisky et al. (2012) studied the biogeography and fungal diversity in the air. They found *Ascomycota* species were represented by 67–85% of the total isolated taxa and taxonomically distributed in four taxonomic classes, namely, *Sordariomycetes*, *Dothideomycetes*, *Eurotiomycetes*, and *Leotiomycetes*, respectively. They represent plant and animal pathogens, symbionts, saprophytes, endophytes and epiphytes, and allergenic taxa (e.g., *Cladosporium* spp., *Aspergillus* spp.).

In the United States, Shelton et al. (2002) evaluated the presence of indoor airborne fungi in 1717 buildings from 1996 to 1998, including hospitals, homes, schools, and industries. They determined *Aspergillus versicolor* as the predominant taxon, followed by *A. flavus*, *A. fumigatus*, and *A. niger*. Studies of Samson (2010) and Flannigan et al. (2011) listed 100 fungal species common in indoor environments. In these lists, *A. fumigatus* and *A. sydowii* were common in the collected house dust. As part of a worldwide survey of the indoor mycobiota, dust was collected from nine countries (Australia, Indonesia, Mexico, Micronesia, New Zealand, South Africa, Thailand, the United Kingdom, and Uruguay). Mycological analyses of samples included the culture-dependent dilution-to-extinction method and the culture-independent 454-pyrosequencing. They found 2717 isolates out of the 7904 belonging to *Aspergillus*, *Penicillium*, and *Talaromyces*, respectively (Visagie et al. 2014). Studies showed that *A. versicolor* is considered as being very common in indoor environments, and recently it has shown to represent a species complex, with nine new species introduced (Jurjević et al. 2012).

The diversity of air mycobiota showed the highest diversity in countries that are also listed as biodiversity hotspots of the world (Myers et al. 2000). This might refer to that the origin of at least a considerable proportion of these species isolated from house dust is from outdoors. However, the prevalence of specific species commonly isolated from indoor surveys suggests that the indoor environments do select for the growth of specific species. In addition, much of the metagenomics diversity may come from transient, dormant, or dead spores (Visagie et al. 2014). Júnior et al. (2012) studied biodiversity of *Aspergillus* spp. and *Penicillium* spp. residing in libraries in Brazil. The genus *Aspergillus* was highlighted as one of the principal airborne fungi present in indoor environments. *Aspergillus* spp. was identified in 1,277 (89.6%) samples and *Penicillium* spp. in 148 (10.4%). The dry period exhibited a greater number of isolates of the two taxa. Frequency of species of 34 taxa of genus *Aspergillus* (anamorph and teleomorph) isolated from library units in the dry (2009) and wet season (2010) in the city of Cuiabá, MT, Brazil, was studied. The taxa belonged to 13 sections. *Aspergillus niger* var. *niger* came first by a recorded 30.2% frequency of occurrence followed by *A. flavus* (19.7%).

In Egypt, Abdel-Azeem and Rashad (2013) studied mycobiota of outdoor air that can cause asthma: a case study from Lake Manzala, Egypt. They isolated a total of 71780 mold and 560 yeast colony-forming units from 600 exposures, and the

isolated taxa were assigned to 28 genera and 43 species. They found that the greater presence of fungal spores occurred in the summer. *Aspergillus niger*, *Cladosporium cladosporioides*, *Epicoccum nigrum*, *Aureobasidium pullulans*, *Alternaria cheiranthi*, *Penicillium chrysogenum*, *Aspergillus fumigatus*, and *Alternaria alternata* were the predominant species. They found that *Aspergillus*, *Cladosporium*, *Penicillium*, and *Alternaria* that had the greatest frequencies in air of Lake Manzala are strongly associated with allergic respiratory disease, especially asthma, in Port Said and Ismailia governorates.

4.2.9 Decaying Wood and Mummies

Wood deterioration by fungi may occur from several sources. These include the following: surface molds that cause localized discoloration; stain fungi that penetrate deep into the sapwood causing blue, gray, green, red, or other dark coloration; and wood-destroying fungi that decompose cell wall polymers (Blanchette 1998). Many ascomycetous fungi such as *Aspergillus nidulans*, *A. fumigatus*, and *A. oryzae*, *Magnaporthe grisea*, *Neurospora crassa*, and *Fusarium gramineum* have a higher number of cellulases, with 34–44 hemicellulase-encoding genes and even 1–5 of the most efficient cellobiohydrolases (Hatakka and Hammel 2010). Research on microbial and enzymatic degradation of wood and wood components has provided a great deal of information that has been useful in helping to protect and conserve historic and archaeological wood. Ascomycetes fungi (anamorphic and teleomorphic) usually cause soft-rot decay of wood with soft brown appearance cracked and checked when dry (Nilsson et al. 1989; Blanchette 1995). Two forms of soft rots were described by Blanchette (1995): type I consisting of biconical or cylindrical cavities that are formed within secondary walls and type II that refers to an erosion form of degradation. The knowledge about lignocellulose degradation by ascomycetes is rather limited in comparison with other basidiomycetous fungi, and very little is known about how they degrade lignin (Nilsson et al. 1989).

Zidan et al. (2006) studied the conservation of a wooden Graeco-Roman coffin box, and they isolated *Paecilomyces variotii*, *Penicillium aurantiogriseum*, *Aspergillus niger*, *Aspergillus flavus*, *Aspergillus terreus*, *Emericella nidulans*, and *Mucor racemosus.* These fungi were found in various parts of the coffin box, and their growth rate varied from one part to the other. In Latvia, during a period from 1996 to 2007, a total of 300 private and public buildings as well as more than 20 cultural monuments had been inspected regarding the damage by wood-decay basidiomycetes and wood-discoloring microfungi (Irbe et al. 2009). Wood-decay fungi in constructions occurred in 338 cases. Brown-rot damage occurred more frequently (78.1%) than the white-rot (21.9%). Wood-discoloring fungi (molds and blue stain) on construction and decorative materials were recorded in 55 cases where frequent genera were *Penicillium*, *Cladosporium*, *Aspergillus*, and *Trichoderma*.

Aspergillus candidus, *A. ustus*, and *A. terreus* were isolated from two wooden masks dating back to the Greek-Roman period in Egypt (Darwish et al. 2013). Abu Deraz (2014) studied the soft-rot fungi deteriorating archeological wood in Al-Aqsa Mosque, Jerusalem, occupied by Palestine. He isolated *Aspergillus flavus*, *A. fumigatus*, *A. glaucus*, *A. niger*, *A. ochraceopetaliformis*, and *Emericella nidulans.* Both *A. flavus* and *A. niger* showed high frequency of occurrence in all examined samples. Mummies have been widely investigated by phenotypic and molecular techniques particularly the study of ancient bacteria and micromycetes. There are several well-known examples showing the colonization of preserved bodies by opportunistic fungi, such as the case of the restoration of the body of Ramses II, performed in Paris in 1976–1977. The mummy showed a dense fungal population with species belonging to the genera *Aspergillus* and *Penicillium* (Mouchaca 1985). In his study *Mouchacca isolated 21 species and one variety of Aspergillus from debris (D) and abdominal materials (A) of Ramses II mummy. The most common species of D and A were A. niger, A. flavus, A. versicolor, A. sydowii, A. amstelodami and A. restrictus.* Aspergilli also dominated the microbial communities of the air and dust of the Egyptian mummy chamber at the Baroda Museum in India (Arya et al. 2001).

Additionally, saprophytic fungi belonging to the genera *Monilia*, *Penicillium*, *Alternaria*, *Aspergillus*, *Rhizopus*, and *Chrysosporium* as well as saprophytic bacteria of the genus *Bacillus* were isolated from a mummy from the collection of the Archaeological Museum in Zagreb, Croatia (Čavka et al. 2010). Fungal genera more related to the mummy materials were *Botryotinia*, *Gibberella*, *Didymella*, *Fusarium*, *Verticillium*, *Tritirachium*, *Coprinus*, and *Coniosporium* (Piñar et al. 2013). Microscopic fungi were isolated from different materials including muscles, bones, skin, and funeral clothes from the mummified human remains of three members of the Kuffner's family and from the surrounding air environments in Slovakia by Šimonovičová et al. 2015. Their hydrolytic abilities such as cellulolytic, lipolytic, and proteolytickeratinolytic were also assessed. The most isolated fungi, from human remains, belonged mainly to the species of *Aspergillus* (*A. candidus*, *A. calidoustus*, *A. fumigatus*, *A. niger*, *A. sydowii*, *A. terreus*, *A. ustus*, *A. venenatus*, *A. versicolor*, *A. westerdijkiae*).

4.2.10 Stones

The tiny pores and cracks in rocks which buffer microbial communities from a number of physical stresses, such as desiccation, rapid temperature variations, and UV radiation, are defined as endolithic environment. The diversity of microorganisms in these ecosystems gained a considerable attention, but few culture-independent studies have been carried out on the diversity of fungi to date. Raghukumar et al. (1992) studied the endolithic fungi from deep-sea calcareous substrata from calcareous animal shells at 100–860 m depth in the Bay of Bengal.

They found that conidia of an isolate of *Aspergillus niger* obtained from intertidal calcareous shells did not germinate above 1 atm. Up to 512 Mu calcium was leached out upon growth of *A. restrictus* on 1 g of calcareous shell substrata at 100 atm. in 25 days.

Diversity of endolithic fungal communities in dolomite and limestone rocks from Nanjiang Canyon in Guizhou Karst Area, China, was studied by Tang et al. (2012). The most common genus in the investigated carbonate rocks was *Verrucaria*. *Aspergillus* and *Penicillium* were also identified from the rock samples. The diversity of culturable fungi associated with six species of healthy South China Sea gorgonians were investigated using a culture-dependent method followed by analysis of fungal internal transcribed spacer sequences (Zhang et al. 2012). A total of 121 fungal isolates were belonged to 41 fungal species from 20 genera. Of these, 30 species and 12 genera are new records for gorgonians, and the genera *Aspergillus* and *Penicillium* were the most diverse and common. Fourteen *Aspergillus* were isolated: they were *Aspergillus carneus*, *A. flavus*, *A. fumigatus*, *A. gracilis*, *A. insulicola*, *A. niger*, *A. nomius*, *A. ochraceopetaliformis*, *A. penicillioides*, *A. sclertoiorum*, *A. sydowii*, *A. terreus*, *A. tubingensis*, and *A. versicolor*. Abu Deraz (2014) recovered seven species of endolithic fungi from archeological stones of Al-Aqsa Mosque, Jerusalem (occupied by Palestine). Surface-sterilized stones were incubated on modified Czapek's medium supplemented with calcium carbonate, as sole carbon source, as described by Kurakov et al. (1999). Five species of genus *Aspergillus* were common: they were *Aspergillus flavus*, *A. fumigatus*, *A. niger*, *A. terreus*, and *Emericella nidulans*.

4.2.11 Human

The fungal biota in an environment (mycobiome) is an important component of the human microbiome (Cui et al. 2013). Every human has fungi as part of their microbiota; however, the impact of fungi on human health is significant, especially as a reservoir for pathogenic fungi when the host is compromised and as a potential cofactor in inflammatory diseases and metabolic disorders (Huffnagle and Noverr 2013). Findley et al. (2013) studied the skin mycobiota of ten healthy Americans, six men and four women. Genera, including the potentially medically significant *Candida*, *Chrysosporium*, and *Cryptococcus*, and otherwise unnamed dermatophytes assigned to the *Arthrodermataceae*. Common saprobic genera such as *Aspergillus*, *Cladosporium*, *Epicoccum*, *Leptosphaerulina*, *Penicillium*, *Phoma*, and *Rhodotorula* were also frequently detected or isolated.

A survey on oral fungal genera has been carried out by Ghannoum et al. (2010). They found that *Candida* and *Cladosporium* were most common, present in 75% and 65% of participants, respectively. The fungi of the oral cavity were previously believed to be few and relatively non-diverse based on culture-dependent or genus-/species-focused culture-independent methods of identification. In contrast with fun-

gal genera associated with local, oral, and invasive diseases, they were *Aspergillus*, *Cryptococcus*, *Fusarium*, and *Alternaria*, indicating that these genera are present in the oral microbiome even during healthy state (Ghannoum et al. 2010). Different studies (Schuster 1999; Salonen et al. 2000; Williams and Lewis 2000

; Jabra-Rizk et al. 2001; Seed 2015) reported different genera of yeasts and filamentous fungi, e.g., *Candida*, *Saccharomyces*, *Penicillium*, *Aspergillus*, *Geotrichum*, and *Scopulariopsis*, and the abundance and presence of *Candida*, *Aspergillus*, and *Fusarium* were recorded among the HIV-infected.

A large number of new emerging pathogens have been described, besides the most prevalent and well-known fungal pathogens such as *Candida albicans* and *Aspergillus fumigatus* (Horré et al. 2010; Marguet et al. 2012). The lung mycobiome of healthy people is comprised of various genus and species principally controlled by environment agents including *Aspergillus* species (van Woerden et al. 2013 and Underhill and Iliev 2014). Aspergilloses are commonly caused by the *fumigatus*, *flavus*, and *niger* groups of genus *Aspergillus*. Other groups rarely act as agents of pulmonary disease, but it is assumed that any species can cause hypersensitivity reactions (Londero and Guadalupe-Cortés 1990). *Aspergillus* species responsible for pulmonary aspergillosis were *A. amstelodami*, *A. candidus*, *A. carneus*, *A. fischeri*, *A. flavus*, *A. fumigatus*, *A. glaucus*, *A. niger*, *A niveus*, *A. phialiseptus*, *A. restrictus*, *A. sydowii*, *A. terreus*, and *A. versicolor* (Londero and Guadalupe-Cortés 1990 and Júnior et al. 2012). Finally, common taxa of *Aspergillus* and human biome are represented by *A. fumigatus*, *A. flavus*, *A. niger*, and *A. versicolor*.

4.2.12 Fossils

Today there are reports of representatives of many different groups of fungi in amber because the translucent nature of the matrix makes it relatively easy to determine even very delicate features useful in systematics, as well as those useful in determining interactions with other organisms (Taylor et al. 2015). Some examples including genus *Aspergillus* have been recorded. Thomas and Poinar (1983) described *Aspergillus* from a piece of Eocene amber originating from the Dominican Republic as *Aspergillus janus*. *A. collembolorum* is a novel species introduced in 2005 by Dörfelt and Schmidt when they studied a piece of Baltic amber (Tertiary, Eocene) which contains an inclusion of a springtail (*Collembola*). The studies discussed above reflect that the genus *Aspergillus* can be characterized with high adaptability to various ecological environments as shown in Fig. 4.1. However, it is important to mention that the results of any study aimed at the examination of *Aspergillus* biodiversity should always be evaluated in the context of the developmental stage of *Aspergillus* taxonomy and the species identification methods available at the time of the publication of the respective paper.

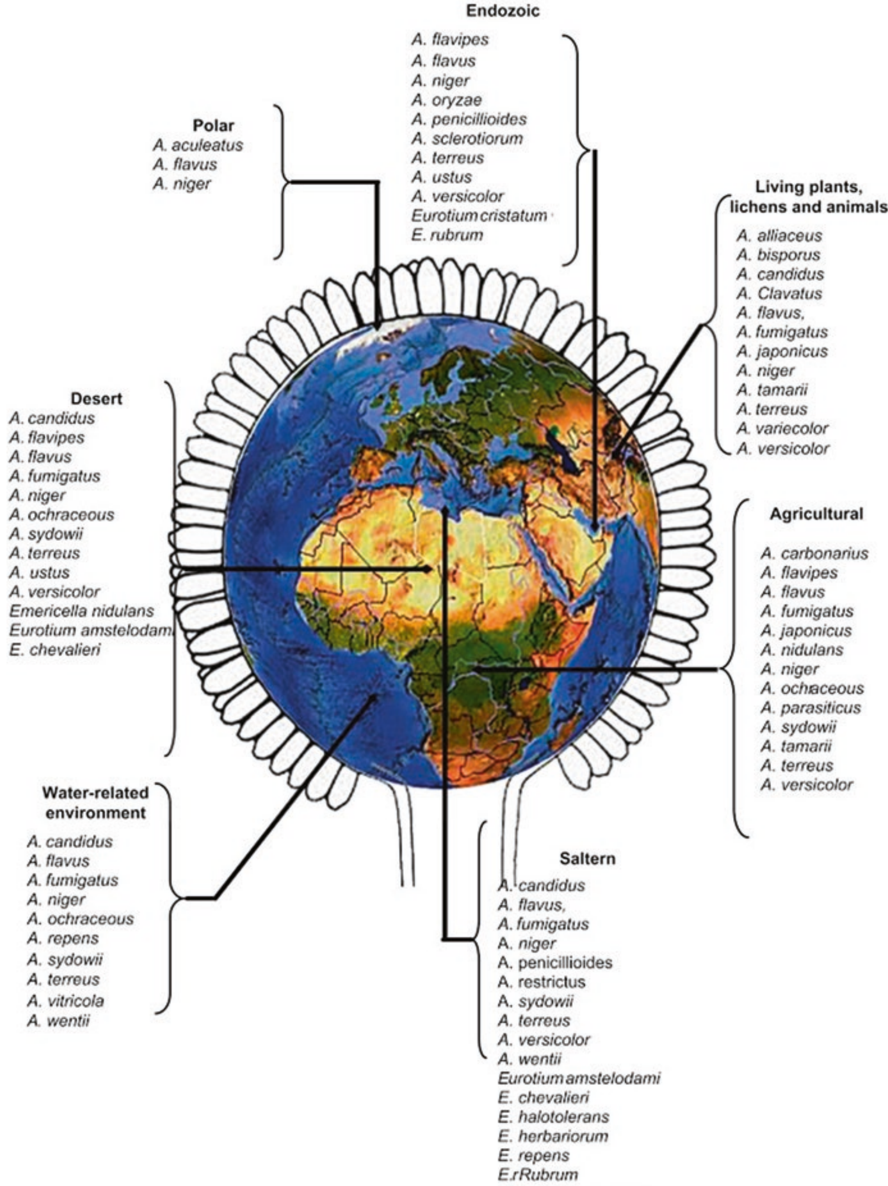

Fig. 4.1 Distribution of *Aspergillus* species among the different biomes of the world as proposed by Abdel-Azeem and Salem

4.3 Biotechnological Industrial Applications

4.3.1 Metabolite Production

Microbiota produces several compounds that they use for their survival, called metabolites, and are the intermediates and products of metabolism. The term metabolite is usually restricted to low-molecular-weight molecules, and they have various functions, including cell signaling, stimulatory and inhibitory effects on enzymes, catalytic activity of their own, defense, and interactions with other organisms (e.g., pigments, odorants, and pheromones). Metabolites are divided into primary and secondary in which a primary metabolite is directly involved in normal growth, development, and reproduction. Many secondary metabolites have proved invaluable as antibacterial or antifungal agents, anticancer drugs, cholesterol-lowering agents, immunosuppressants, antiparasitic agents, herbicides, diagnostics, and tools for research (Yadav et al. 2017a, b). Some of these have been found to play a pivotal role in treatment or prevention of a multitude of biological disorders, many of which did not have any cure until these products were discovered (Vaishnav and Demain 2010; Hansson 2013). Aspergilli have been successfully employed in the biotechnology sector due to their great production of organic acids and extracellular enzymes (Khan et al. 2014). In this part of chapter, important aspects of their role in providing secondary metabolites will be described along with their biotechnological perspectives.

4.3.1.1 Polyketides

The history of polyketides started when James Colie synthesized orcinol at London University, in 1893 (Khan et al. 2014). In 1950 the Australian organic chemist Arthur Birch proved that polyketides are biosynthesized by acetate units with the help of nuclear magnetic resonance (NMR), which was evolving in those years. In 1955 Birch published the work on 6-methyl salicylic acid released by a fungus, *Penicillium griseofulvum* (Birch et al. 1955). Polyketides are the most abundant secondary metabolites in fungi, also being produced by plants and bacteria. The compound is synthesized by the action of polyketide synthase (PKS), which is similar to fatty acid biosynthesis. These natural organic compounds have a complex chemical structure and have played important roles in the pharmaceutical field. Important antibiotics are polyketides, such as doxycycline, clarithromycin, and erythromycin. Regarding the production of polyketides by *Aspergillus*, aflatoxin and lovastatin are among the more well-known and will be described here in more detail (Keller et al. 2005). Ongoing research has also revealed more compounds that might be of interest (Figs. 4.2 and 4.3).

Fig. 4.2 Typical fungal polyketides

Fig. 4.3 Bioactive fungal polyketides

4.3.1.1.1 Lovastatin

Lovastatin (Fig. 4.2) is a potent 3-hydroxymethylglutaryl-CoA (3-HMG-CoA) inhibitor, discovered in the late 1970s at Merck Research Laboratories in the fermented broth of *Aspergillus terreus*, used in the treatment of hypercholesterolemia (Alberts et al. 1980; Tobert 2003). However, since its discovery, research has been performed to optimize the production of this polyketide metabolite such as medium composition, aeration conditions, fungal morphology, and broth rheology. More recently Osman et al. (2011) and Bizukojc et al. (2012) have explored the pH in the production of lovastatin (Fig. 4.4). It presented anticoagulant or anti-inflammatory and antibacterial properties (Campbell et al. 1985; Rehse and Lehmke 1985; Antane et al. 2006; Xu et al. 2013). In another recent study, Gao et al. (2013) isolated aspulvinones from *A. terreus* in a mangrove in Fujian, a Chinese province, with anti-influenza A viral (H1N1) activity.

4.3.1.1.2 Aflatoxin B1

Several *Aspergillus* fungi have contributed to the field of biotechnology. However, toxic metabolites are also produced. It can impose a threat to other microorganisms as well as to humans, among them *Aspergillus flavus*. Although first described in 1809, the fungus that secretes aflatoxin came into the limelight in the 1960s, causing

Fig. 4.4 Lovastatin production pathway after Jahromi et al. (2012)

the death of over 100,000 turkey poults in London due to the contamination of the peanuts with which the turkeys had been fed (Keller et al. 2005; Bhatnagar-Mathur et al. 2015). Aflatoxins are nondigestible by animals and end up in the meat. They are also heat- and freeze-stable and remain indefinitely in the food. The toxin (Fig. 4.5) has a high impact on human health worldwide, causing aspergillosis and slowing the recovery rate from protein malnutrition (Amare and Keller 2014). The endeavors to combat aflatoxin in crops with biotechnological tools have been recently reviewed by Bhatnagar-Mathur et al. (2015).

Fig. 4.5 Chemical structure of aflatoxins

4.3.1.1.3 Ochratoxin

Ochratoxin is a polyketide derivative and is very important in the fungal biotechnological process due to its characteristics. Ochratoxin is a mycotoxin found in food and beverages that exhibits nephrotic effects and can, potentially, be associated with human carcinogenesis. Ochratoxin is known for contaminating grapes and wines. Besides that, the compound has toxicological effects like nephrotoxicity and hepatotoxicity (Crespo-Sempere et al. 2014). Because of its importance, work has still to be done in the direction of understanding better the gene expression and ochratoxin production, as reported by Castellá et al. (2015), with *Aspergillus niger*, leading the authors to suggest that using real-time polymerization chain reaction (PCR) would allow early detection of expression of the gene before accumulation of the toxin in food and the application of measures to prevent its biosynthesis (Fig. 4.6).

4.3.2 Enzymes

Fungi are great producers of enzymes and have contributed enormously to enable and facilitate industrial processes. From food to pharmaceutical products and chemical goods, these enzymes have proven their importance in our everyday lives. The aspergilli are specially required in the field, accounting for more than 300 species (Abdel-Azeem et al. 2016). *Aspergillus oryzae* and *A. niger* have fundamental importance as they are on the list of generally recognized as safe (GRAS) of the Food and Drug Administration (FDA) in the United States (Contesini et al. 2010).

Fig. 4.6 Chemical structures of ochratoxin A (dark blue, phenylalanine part; red, dihydroisocoumarin ring; green, acidic hydrogens), B, and C. The highlighted structures are characteristic to the three different ochratoxin molecules (light blue)

4.3.2.1 Lipases

Lipases (triacylglycerol acyl hydrolases, EC 3.1.1.3) are natural catalysts of the hydrolysis of triacylglycerol into di- and monoacylglycerols, fatty acids, and glycerol at an oil-water interface, a phenomenon known as interfacial activation. However, under certain conditions, they are also able to catalyze synthetic reactions. The most reported of the reactions carried out by these enzymes are hydrolysis, acidolysis, alcoholysis, amylolysis, esterification, and interesterification. The current updated applications of lipase are in detergents (removal of oil stains from fabrics), in food industry (attainment of functional phenols and aroma ester synthesis), in pharmaceutical industry (kinetic resolution of ketoprofen and kinetic resolution of diltiazem intermediate), and in fuel industry as in biodiesel production (Contesini et al. 2010; Sahay et al. 2017; Yadav et al. 2016). *Aspergillus ibericus*, *A. niger*, and *A. uvarum* were selected as suitable microfungi to produce lipase in SSF (Salgado et al. 2014). The results, as suggested by the workers, may have potential application in the simultaneous management and valorization of olive mill and wineries wastes.

4.3.2.2 Laccase

Laccase (EC 1.10.3.2) is a copper-containing oxidase enzyme and was described for the first time by Hikorokuro Yoshida at the end of the nineteenth century (Yoshida 1883). The Japanese researcher extracted the enzyme from the lacquer tree *Rhus vernicifera*. In 1885 Gabriel Bertrand then found that laccase is a metalloprotein.

However, at that time Prof. Bertrand pointed out manganese as the metal due to an insufficiently purified enzyme (Lehn et al. 1986). The metal associate is in fact copper, as later reported by Keilin and Mann (1939). Laccase is predominantly present in plants and fungi but is also found in insects and bacteria (Kunamneni et al. 2007). Importantly, fungal laccases have higher redox potential than that from bacteria or plant sources. They are involved in the degradation of lignin and removal of toxic phenolic compounds. Apart from that, laccases might also be involved in the synthesis of melanin (dark polymers produced against environmental stress) (Kunamneni et al. 2007). Laccases from fungi have been reviewed by Thurston (1994) and Mayer and Staples (2002), who also pointed out the innumerous uses of the enzyme, and Giardina et al. (2010), referring to the genetic regulation aspects. The range of applications of this enzyme is broad and encompasses several industrial sectors, thereby being part of many important processes, from ethanol production to drug analysis, wine clarification, trichlorophenol, bioremediation, herbicide degradation, and decolonization of dyes and in the paper industry and in the textile industry, to name a few (Mayer and Staples 2002; Kunamneni et al. 2007).

Many aspergilli, such as *A. nidulans*, *A. oryzae*, *A. niger*, and *A. fumigatus*, have been reported to produce laccases (Thurston 1994; Scherer and Fischer 1998). Mander et al. (2006) explored *A. niger* and *A. nidulans* to produce laccase and used the enzyme as a protein reporter. Studies like the one conducted by Ramos et al. (2011) are seeking to improve parameters and optimize the use of laccase produced by *Aspergillus* for biotechnological purposes. Because the use of laccase in the abovementioned processes requires a large amount of the enzyme at low cost, researchers have made efforts to optimize the fermentation process, thereby enabling the production of the enzyme in a more affordable industrial scale (Couto and Toca-Herrera 2007).

4.3.2.3 Tannases

Tannin acyl hydrolase (EC 3.1.1.20) catalyzes the hydrolysis of ester and depside bonds in hydrolyzable tannins such as tannic acid, methyl gallate, ethyl gallate, n-propyl gallate, and isoamyl gallate, releasing glucose and gallic acid. Gallic acid catalyzes the second step in the degradation of tannic acid (Lal and Gardner 2012). Tannase has been reported to be produced in several fungi. Despite white-rot fungi being good laccase producers, aspergilli also present interest regarding the obtaining and application of the enzyme (Couto and Toca-Herrera 2007; Kumar et al. 2007; Paranthaman et al. 2008; Costa et al. 2013; George and Ong 2013). In particular *A. niger* has been used to produce tannase (Pinto et al. 2001) and compare the production in solid-state and submerged fermentation (Aguilar et al. 2001; Mata-Gomez et al. 2009), using agricultural residues as alternative substrates in different fermentation methods (Hamdy and Fawzy 2012).

As reviewed by Lal and Gardner (2012), fungal tannase is used in many industrial applications including clarification of fruit juice (Shrivastava and Kar 2009); detannification of food (Boadi and Neufeld 2001); preparation of food preservatives

(Belmares et al. 2004); high-grade leather tanning (Lekha and Lonsane 1997); clarification of beer and wines (Bajpai and Patil 2008); manufacture of coffee-flavored drinks (Anwar and Imartika 2007); manufacture of instant tea (Lekha and Lonsane 1997); production of gallic acid, which is used for the synthesis of trimethoprim (Yu et al. 2004); treatment of green tea to inhibit the carcinogenic and mutagenic effects of N-nitrosamines; stabilization of malt polyphenols (Lekha and Lonsane 1997); and improving color stability and additional organoleptic properties. In animal feeding, tannase is used to reduce the antinutritional effects of tannins and improve animal digestibility. Tannase is also utilized for bioremediation of effluents from tanneries. In addition, tannase is used as a sensitive analytical probe for determining the structure of naturally occurring gallic acid ester. Ma et al. (2014) explored *Aspergillus ficuum* production of tannase in SSF. The authors also performed studies to optimize the process, obtaining relatively high yields of the enzyme using wheat bran as substrate.

4.3.2.4 Pectinases

Pectinases are enzymes that break down pectin, a structural heteropolysaccharide found in primary plant cell walls of terrestrial plants, cereals, fibers, fruits, and vegetables. They were first isolated and described in 1825 by Henri Braconnot (Anisa et al. 2013; Kohli and Gupta 2015). Commonly referred to as pectic enzymes, they comprise pectin lyase, pectozyme, and polygalacturonase. One of the most studied and widely used commercial pectinases is polygalacturonase. It is useful because pectin is the jellylike matrix which helps cement plant cells together and in which other cell wall components are embedded. Therefore pectinase enzymes are commonly used in processes involving the degradation of plant materials, such as speeding up the extraction of fruit juice, including apples and sapota.

Fungi are preferred by the industry as a source of pectinase, since they secrete the enzyme in the culture medium, facilitating its recovery (Soares et al. 2012). They can be extracted from fungi and the most popular fungus used to obtain pectinase is *A. niger*. The fungus produces these enzymes to break down the middle lamella in plants so that it can extract nutrients from the plant tissues and insert fungal hyphae. If pectinase is boiled, it is denatured (unfolded), making it harder to connect with the pectin at the active site and produce as much juice (Debing et al. 2006).

Nowadays the enzyme makes up a quarter of the global market of food enzymes and about 10% of the global market (Anisa et al. 2013; Kohli and Gupta 2015; Kumar et al. 2017). Therefore, studies such as those conducted by Sandri et al. (2011) and Anisa et al. (2013) still attempt to optimize the production of pectinases, exploring different aspergilli and substrates as well as comparing different fermentation methods. It has been reported that SSF leads to higher enzymatic production than submerged fermentation (Maheshwari 2003). Several substrates are known to be used in pectinase production, such as wheat bran, rice straw, and Tween 80, which have also been applied to produce pectinase (Debing et al. 2006). In this direction, Esawy et al. (2013) studied the production of pectinase from *A. niger*

using Egyptian citrus peels as the carbon source. In order to optimize its results, the authors immobilized the enzyme in polyvinyl alcohol sponges. The researchers observed superiority in all properties analyzed using pectinase immobilized over the free enzyme pointing out its suitability for orange juice clarification. Regarding the substrates, wheat bran and potato starch have been employed with success. Another report by Durairajan and Sankari (2014) described maximum production of the enzyme from *A. niger* using banana peel rather than orange and pineapple peel. Other substrates are also commonly used for the production of pectinases, such as sugar cane bagasse and citrus peels.

4.3.2.5 Proteases

Proteases have important roles in baking, brewing, the production of various Oriental foods such as soy sauce and miso, meat tenderization, and cheese manufacture. The first contact of humans with protease activities occurred when we started producing milk curd. Desert nomads from the East used to carry milk in bags made of goat stomach. After long journeys, they realized that the milk became denser and sour, without understanding the process's cause. Curds thus became a food source and a delicacy. Renin, an animal-produced enzyme, is the protease which caused the hydrolysis of milk protein (Soares et al. 2012). The use of protease includes food processing, detergents, dairy industry, animal nutrition, paper and pulp, textiles, and leather making (Negi and Benerjee 2006; Chutmanop et al. 2008; Hamada et al. 2013).

Nowadays proteases account for nearly 60% of the enzyme market, which raises interests in optimizing its production and obtaining better-quality enzymes (Jinka et al. 2009).The substrate is one of the most important parameters in enzymatic production as it is related to the final cost of the product. Taking this into account, Negi and Benerjee (2006) produced protease concomitantly with amylase.

Using wheat bran as substrate and SSF as the production method, they reached good amounts of both enzymes from *Aspergillus awamori* in a single bioreactor. Chutmanop et al. (2008) also used SSF to analyze protease production. In that case *A. oryzae* was the chosen fungus and rice bran was explored as a promising substrate due to its large availability in Asian countries, besides being cheaper than wheat bran. The use of rice bran alone was shown to not be interesting, as low porosity prevented sufficient oxygen penetration, resulting in low performance. Yet, the authors found that a mix of rice bran with 25% wheat bran improved substantially the production, indicating a route to obtain proteases at reduced costs. Further studies have explored agro-industrial wastes (De Castro and Sato 2014) and potato pulp as substrates to produce proteases from *A. oryzae*. Siala et al. (2012) studied the production of aspartic protease from *A. niger*. Ongoing research to enhance the process parameters to obtain protease from other aspergilli have been conducted as those employing *Aspergillus clavatus* (Tremacoldi et al. 2004), *Aspergillus fumigatus*, *A. flavus* (Oyeleke et al. 2010), and *Aspergillus foetidus* (Souza et al. 2015).

4.3.2.6 Lactases

Lactases are β-galactosidases, enzymes that catalyze the hydrolysis of lactose into galactose and glucose (Maksimainen et al. 2013). β-Galactosidase is highly important in the dairy industry and in the hydrolysis of lactose into glucose and galactose with an improvement in the solubility and digestibility of milk and its related products. Food with low-lactose contents or lactose-free is thereby obtained (Soares et al. 2012). Thus, a relief for people who suffer from lactose intolerance (estimated at 70% of adults worldwide) as a result of lactase insufficiency or nonexistence in the colon, resulting in abdominal pain, nausea, and diarrhea due to malabsorption of lactose (Ingram et al. 2009; Maksimainen et al. 2013; de Vrese et al. 2015).

β-Galactosidases are also used in reverse hydrolysis to obtain galacto-oligosaccharides (GOS) and used as probiotics in food to stimulate the growth of beneficial bacteria in the colon (Vera et al. 2012; Maksimainen et al. 2013). Regarding its biotechnological production from filamentous fungi, *A. oryzae* has been especially studied for providing the enzyme in sufficient amounts, being commercially available and used in the milk industry (Maksimainen et al. 2013). Research has been conducted to characterize and evaluate its production over the last decades (Friend and Shahani 1982; Corazza et al. 1992; de Vrese et al. 2015). During the last years, researchers have also concentrated in revealing structural details of β-galactosidases, providing information to tune the application of this important enzyme (Ito et al. 2002; Cantarel et al. 2009; Maksimainen et al. 2013).

4.3.2.7 Cellulases

Cellulases comprise enzymes that break the glycosidic bonds of cellulose microfibrils, releasing oligosaccharides, cellobiose, and glucose. Cellulases from fungi have had their properties and production process studied for decades (Hurst et al. 1977; Begum and Absar 2009; Ncube et al. 2012). These hydrolytic enzymes are not only used in food, drug, cosmetics, detergents, and textile industries but also in the wood pulp and paper industry, in waste management, and in the medical-pharmaceutical industry (Bhat and Bhat 1997). In the food industry, cellulases are employed in the extraction of components from green tea, soy protein, essential oils, aromatic products, and sweet potato starch.

Sohail et al. (2009) investigated the production of cellulases from *A. niger* in an attempt to obtain a sufficient amount of β-glucosidase, which is produced in low levels in species of *Tricoderma*, a well-studied system for enzymatic depolymerization of cellulosic material. The results were promising, leading to a moderate to high production of endonuclease and β-glucosidase. The work was carried out in different substrates, namely, grass, corncob, and bagasse.

4.3.2.8 Amylases

Amylases are starch-degrading enzymes that started to be produced during the twentieth century due to their great industrial importance, being responsible for approximately a quarter of the enzyme market (Ratnasri et al. 2014). In fact, they are the most important industrial enzymes with high biotechnological relevance. Their uses range from textiles, beer, liquor, bakery, infant feeding, cereals, starch liquefaction-saccharification, animal feed industries, to chemical and pharmaceutical uses. The species *Aspergillus* and *Rhizopus* are highly important among the filamentous fungus for the production of amylases (Pandey et al. 1999, 2006). The food industry uses amylases for the conversion of starch into dextrin. The latter are employed in clinical formulas as stabilizers and thickeners; in the conversion of starch into maltose, in confectioneries, and in the manufacture of soft drinks, beer, jellies, and ice cream; in the conversion of starch into glucose with applications in the soft drinks industry, bakery, and brewery and as a subsidy for ethanol production; and in the conversion of glucose into fructose, used in soft drinks, jams, and yoghurts (Aquino et al. 2003; Nguyen et al. 2002).

Amylases provide better bread color, volume, and texture in the baking industry. The use of these enzymes in bread production retards its aging process and maintains fresh bread for a longer period. Whereas fungal amylase provides greater fermentation potential, amyloglucosidase improves flavor and taste and a better bread crust color (Soares et al. 2012). *Aspergillus oryzae* has been a producer of amylase as exemplified by the work of Chang et al. (1995) and Kariya et al. (2003), for the purification of amylase. Other groups have explored amylase production in *A. niger* (Hernandéz et al. 2006; Rosés and Guerra 2009) and *A. fumigatus* (Ratnasri et al. 2014). These works have explored the potential of alternative substrates such as sugar cane bagasse, cereal flours, or brewery (supplemented by casamino acids, peptone, and yeast extract) in the production of fungal amylases.

4.3.3 Organic Acids

Organic acids are the most common acids, such as carboxylic acids. Fungal biotechnology is very important for the production of many organic acids. Although the conversion of organic acids can reach as high as 80% in living cells, standing out in productivity terms, some of these compounds occupy a relevant place in industrial production due to economic reasons when using chemical routes for obtaining organic acids (Magnuson and Lasure 2004; Liaud et al. 2014). Organic acids play an important economical role in our contemporary society due to the wide variety of applications they are involved in, from food to pharmaceuticals and chemical processes, moving markets and supporting the advancement of technologies. The production of organic acids benefited highly from the biotechnological knowledge and improvements made during the twentieth century, thanks to many interdisciplinary teams involving biologists, chemists, pharmacists, and engineers, among

other professionals in projects such as the penicillin production. Although organic acids can be found in other microorganisms such as bacteria, it has been in filamentous fungi that they have been produced for many decades due to the high yields and other process advantages discussed in the following paragraphs.

4.3.3.1 Citric Acid

Citric acid was discovered by Karls Scheels in England in 1874 in lemon juice (Vandenberghe et al. 1999; Max et al. 2010). The production of citric acid is the oldest and most thoroughly studied filamentous fungal fermentation, dating back to 1917, when Currie optimized the conditions using a surface cultivation method (Currie 1917), and nowadays most of its production occurs via microbial processes (Max et al. 2010). The critical parameters for citric acid production by *A. niger* were defined empirically and include high carbohydrate concentration, low but finite manganese concentrations, maintenance of high dissolved oxygen, constant agitation, and low pH (Schreferl et al. 1986; Zhang and Roehr 2002). Kareem et al. (2010) explored the potential of pineapple peel as a cheap medium to produce citric acid, resulting in a production of 60.6 mg/kg of pineapple in optimized conditions. Using apple pomace solid waste, citrus waste, brewery spent grain, and sphagnum peat moss, Dhillon et al. (2011) reported that the substrates were suitable for citric acid production by both methods and might offer significant social, economic, and environmental impact.

4.3.3.2 Itaconic Acid

Itaconic acid is obtained from the distillation of citric acid in 1960 by fermentation of carbohydrates by *A. terreus* (Mitsuyasu et al. 2009; Hajian and Yusoff 2015). Itaconic acid has been applied in a numerous range of industries with the larger producers in the world being the United States, Japan, Russia, and China (Global Industry Analysts Inc. 2011). During the 1950s, itaconic acid was used in industrial adhesives. In that period, itaconic acid was used at an industrial scale and large amounts of it were required. It has been employed as a detergent and in shampoos, as well as in plastics, elastomers, fiberglass, and in the coating process of carpets and book covers (Mitsuyasu et al. 2009; Jin et al. 2010). Besides that itaconic acid may also be used as artificial gems and synthetic glasses (Kin et al. 1998). Lately, the applications of the compound have reached the biomedical fields, such as the ophthalmic, dental, and drug delivery fields (Hajian and Yusoff, 2015).

Several studies have focused on improving and optimizing the production of itaconic acid from *A. terreus* in recent years. The biotechnological aspects involved in the metabolic pathways of itaconic acid and the production process parameters have been reviewed by Klement and Büchs (2013). Regarding the production, El-Imam and Chenyu (2014) obtained itaconic acid using oil by-product jatropha curcas seed cake, while Li et al. (2011), Huang et al. (2014), and van der Straat et al. (2014) studied the itaconic acid production by using genetic engineering techniques.

In this process the relevant pathways have been revealed and new microbial production platforms designed, contributing to an enhanced production of itaconic acid. Furthermore, the reduction of its production costs is an important aspect for itaconic acid producers, either by optimizing processes or by using cost-favorable raw materials.

4.3.3.3 Kojic Acid

Kojic acid (5-hydroxy-2-(hydroxymethyl)-4-pyrone; KA) is an organic acid secreted by several species of *Aspergillus* such as *A. oryzae*, *Aspergillus tamarri*, *Aspergillus parasiticus*, and *A. flavus* (Bentley 2006). The name KA was derived from "Koji," a fungus or starter inoculum used in Oriental food such as sake, shoyu, miso, and vinegar (Terabayashi et al. 2010; Chaudhary et al. 2014). KA is used as a food additive, antibiotic, antioxidant (Bentley 2006), and a skin whitening agent in the cosmetic industry and in medicine, for the treatment of chloasma (Terabayashi et al. 2010), as antitumor agent (Tamura et al. 2006), and as radioprotective agent (Emami et al. 2007). Due to its wide range of applications, KA has been targeted by researchers to discover the biosynthesis pathways in filamentous fungi (Ariff et al. 1996; Futamura et al. 2001; Rosfarizan et al. 2002). Terabayashi et al. (2010) disclosed information about the genes involved in KA biosynthesis in *A. oryzae*. Using DNA microarray technique, the workers found two genes that might be involved in the biosynthesis process, giving insights into the genetic regulation of KA production. Other works have been related to the production methods of KA and bioreactors construction as described by Ogawa et al. (1995) and Wakisaka et al. (1998) in membrane-surface liquid culture (MSLC).

4.3.3.4 Gluconic Acid

Gluconic acid is produced from glucose. In this glucose oxidase catalysis process, the dehydrogenation reaction leads to its production (Ramachandran et al. 2006). It had already been produced in 1870 (Rohr et al. 1983; Ramachandran et al. 2006), being found later by Molliard (1922) in *A. niger*. Since then many researchers have studied the conditions and processes that would lead to better yields. Gluconic acid production by fermentation of glucose using *A. niger* is a mature bioprocess with literature reporting highly efficient processes dating back to 1940 (Moyer et al. 1940). Gluconic acid has applications in the food industry, as in meat and dairy products, baked goods, flavoring agent, and reducing fat absorption in doughnuts (Ramachandran et al. 2006). Although with a market smaller than that of citric acid, gluconic acid finds its place, as well as its derivatives, such as sodium, calcium, and iron gluconate, which is used for dietary supplements, in the pharmaceutical and textile industries (Ramachandran et al. 2006). For that the fungi most commonly used is *A. niger*. Even though several factors influence microbial fermentation, it is believed that oxygen availability and the pH of the medium are key parameters to be addressed. Studies concentrate in

exploring the fermentation processes, as well as alternatives such as cheaper raw materials, enzymatic immobilization, and molecular biology tools, so that the production can be optimal and the results the best possible (Roukas 2000; Ikeda et al. 2006; Ramachandran et al. 2008; Lu et al. 2015; Shi et al. 2015).

4.3.4 Antioxidant

Fungi are remarkably a diverse group including approximately 1.5 million species, which can potentially provide a wide variety of metabolites such as alkaloids, benzoquinones, flavanoids, phenols, steroids, terpenoids, tetralones, xanthones, and anthraquinones. Over the past years, more than 180 *Aspergillus* strains have been isolated from a host of terrestrial ecological niches, and they provide a steady stream of diverse small molecules. Aflatoxin pathway, as a main biosynthesis pathway in the genus of *Aspergillus*, can produce abundant metabolites, which always show antioxidant activity and cytotoxicity (Guravaiah et al. 2018; Wu et al. 2018). Free radicals and oxidants play a dual role, since they can be either harmful or helpful to the body. They are produced continuously in all cells as part of normal cell metabolism in situ or from environmental sources (pollution, cigarette smoke, radiation, medications, etc.), which could generate oxidative stress, a deleterious process that plays a major part in the development of chronic and degenerative diseases, such as arteriosclerosis, diabetes, and cancer initiation, and implicated in the aging process. Therefore, antioxidant therapy represents a promising treatment for the effects of oxidative stress acting as radical scavengers and inhibits lipid peroxidation and other free radical-mediated processes protecting from several diseases. However, the growing concern about potential health hazards caused by the use of synthetic antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), and tert-butylhydroquinone (TBHQ) in food products has led to the scrutiny of natural antioxidants. In view of these health concerns, finding safer, more effective, and economic natural antioxidants is highly desirable (Dewi et al. 2012).

In consequence, attention has been focused on the fungi which are of great biotechnological interest in the fermentative processes that culminate in the production of secondary metabolites. Filamentous fungi produce a diverse array of secondary metabolites that have a tremendous impact on society and are exploited as antioxidants serve as the defensive factor against free radicals in the body including *Aspergillus* sp. Researchers focused to screen and expand the spectrum of fungi having antioxidant potentials and to optimize the culture conditions to enhance their activities in pharmaceutical applications, in addition to safety considerations (Daljit Singh Arora and Chandra 2010a).

The fungal strain *Aspergillus versicolor* has been proven to be a rich source of diverse secondary metabolites with novel structures and interesting bioactivities. Novel and bioactive compounds resulted in the isolation of an extract of cultured *Aspergillus versicolor* fungus that were new xanthone, oxisterigmatocystin D (1), and, a new alkaloid, aspergillusine A (13), along with another three known xanthones

Fig. 4.7 Novel and bioactive compounds resulted in the isolation of an extract of cultured *Aspergillus versicolor* fungus (Wu et al. 2018)

(2–4), eight known anthraquinones (5–12), and four known alkaloids (14–17) (Fig. 4.7) (WU et al. 2018). *Aspergillus terreus* is one such potential candidate offering a better scope for the production and easier downstreaming of bioactive compounds. Terreic acid and terremutin from ethyl acetate extract of *A. terreus* exhibited significant antioxidant activity (Daljit Singh Arora and Chandra 2010a; Dewi et al. 2012). *Aspergillus niger* strains were inoculated on cereals, beans, and their non-useful parts as fermentation substrates, and the fermentations were extracted with ethyl acetate. Antioxidative activities of extracts from fermentations of soybean, soybean curd refuse, polished rice, rice bran, and wheat bran were obviously greater than those of the unfermented matters. *Aspergillus niger* A-12 produced an antioxidative and synergistic compound (J) with tocopherols. On the basis of the HPLC pattern and UV-VIS absorption spectra, the strains AHU 7008, AHU 7362, IFO 31125, and IFO 8877 belonging to the *A. niger* group might produce the antioxidative compound (J) or its homologues (Kawai et al. 1994).

Aspergillus melleus URM 5827 produced tannase (tannin acyl hydrolase EC 3.1.1.20 is an enzyme that catalyzes the hydrolysis of ester and depside bonds present in hydrolysable tannins, and the reaction's final products are glucose and gallic acid),

using achachairu seeds via solid-state fermentation (SSF), which improved antioxidant potential of green tea by approximately 85% when compared to the control (Liu et al. 2017). Phenolic compounds in the ethyl acetate extracts of *Aspergillus* PR78 and *Aspergillus* PR66 were able to scavenge DPPH, ferrous, and nitric oxide ion and have reducing potential in addition to their ability to chelate metals such as iron which indicates their act as potent antioxidants especially *Aspergillus* PR78 which was equally effective as that of commonly used antioxidant standard, ascorbic acid (Daljit Singh Arora and Chandra 2010b). Several antioxidants such as 3,3-di-OH terphenyllin, 3-OH terphenyllin, and candidusin B are obtained from the extracts of *Aspergillus candidus* CCRC 31543 which is reported to have scavenging effects on DPPH radicals (Yen et al. 2003). The optimal conditions are crucial to be studied; as for *Aspergillus oryzae*, the optimal antioxidant extraction conditions were 65.3 °C and 73.1% ethanol for maximum total phenolic concentration and 61.6 °C and 60% ethanol for maximum DPPH radical scavenging activity (Wardhani et al. 2010). When *Aspergillus sojae* and *Aspergillus oryzae* were incubated in a medium including fish oil or fish waste, it had a relatively great effect. In accordance, mackerel waste fermented with *Aspergillus terreus*, *Aspergillus oryzae*, and *Aspergillus flavus* inhibited autoxidation of fish lipid (Kawai et al. 1994).

Nevertheless, safety consideration is the main pillar for applications; as for *Aspergillus fumigatus* extract, bioactive compounds were toxicity evaluated and proved to be neither cytotoxic nor mutagenic (Arora and Chandra 2011). Further studies are required to gain a better understanding of optimal fermentation conditions, extraction methods, and the structure and safety of antioxidants and bioactive metabolites produced by *Aspergillus* sp. fungi, which will be helpful in their biotechnological mass production in the near future as the upraised data highlighted their significance as new sources of natural antioxidants.

4.3.5 Pigments

Pigments from natural sources have been obtained since longtime and with time and interest in production of natural colorants have been increased due to toxic effects of synthetic ones. Natural pigments like carotenoids, flavonoids (anthocyanins), chlorophylls, phycobiliproteins, betalains, and quinones are common pigments that are in use. Among these, due to the ease of cultivation, extraction, and genetic diversity, fungi and bacteria are most promising. Bacteria and fungi such as *Bacillus*, *Achromobacter*, *Yarrowia*, *Rhodotorula*, *Phaffia*, and *Monascus* produce a large number of pigments. Carotenoids that are yellow, red, and orange are widely used as food and feed supplements and as antioxidants in pharmaceutical industry (Mukherjee et al. 2017). Fungal pigments are secondary metabolites that are sometimes produced due to scarcity in the nutritional value. When the nutritional supply of essential nutrients decreases or there is some disfavoring environmental condition, mycelium produces secondary metabolites (Gupta and Aggarwal 2014). There are some fungi including *Aspergillus*, *Fusarium*, *Penicillium*, and *Trichoderma* that

produce various pigments as intermediate metabolites during their growth (Atalla et al. 2011). Fungal pigments are classified as carotenoids and polyketides. Fungal polyketides are made up of tetraketides and octaketides having eight C2 units forming polyketides chain (Mukherjee et al. 2017).

Anthraquinone is the most common class that is proved to be potentially safe (Mapari et al. 2010). Pigment anthraquinone is widely used in dyestuff industry and most commonly produced by *Trichoderma*, *Aspergillus*, and *Fusarium* (Duran et al. 2002). It is now known that single fungal species can produce mixture of different pigments, having various biological properties. Production of theses pigments plays an important role in fungi. Melanin production helps the fungi to survive in severe environmental stress and helps to cope up with UV light (Abu ElSouad et al. 2015). Similarly, there are many other species of mushrooms that produce various pigments imparting different colors. There are more than 100 pigments that have been reported in fungi, and it holds place after the plants (Fig. 4.8).

Various colors of the fungi are one of the very important characteristics that help in their identification. Green color of *Penicillium*, violet color of *Cortinarius*, and yellow (Chen et al. 1969), orange, and red color of *Monascus* (Feng et al. 2012) are their distinct feature. Their pigments provide them protection against UV light and may also from the bacterial attack. The pigments of fungi differ greatly from higher plants being not possessing chlorophyll or the anthocyanins that impart various colors to flowers. Many of the fungal pigments are quinones or similar conjugated structures. The pigmentation in fungus sometimes varies with its age. As observed in *Penicillium chrysogenum* initially, their colonies appear white in color, and later that changes to blue green (Tiwari et al. 2011).

Quinones are very common polyketide fungal pigments that are produced by following polyketide pathway. As its reduction product usually accompanies quinone, this is not necessary that it will show the color of the fungus from which it has been isolated (Feng et al. 2015). Fumigatin (Fig. 4.9) is isolated from *Aspergillus fumigatus* (Anslow and Raistrick 1938). It was observed that the solution in which

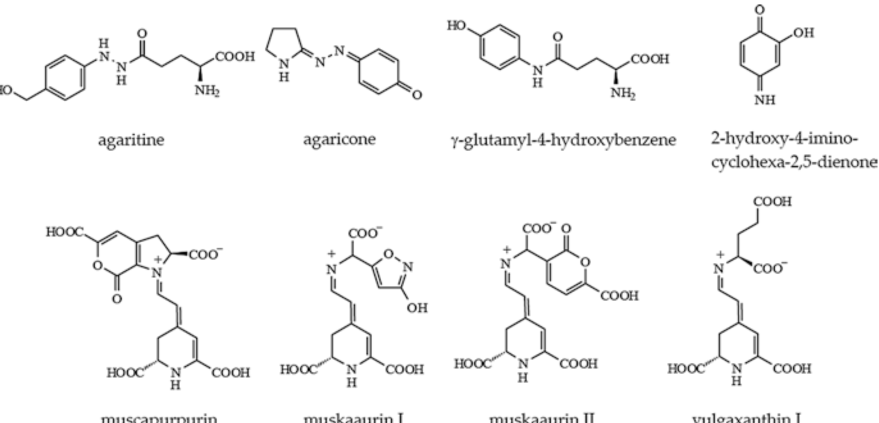

Fig. 4.8 Some pigments reported from higher fungi

Fig. 4.9 Structure of various pigments

Aspergillus fumigatus was grown was initially yellowish brown and later changed its color to purple when treated with alkali (Hanson 2008). Auroglaucin and flavoglaucin (Fig. 4.9) 528 G, according to Mukherjee et al. (2017) are the pigments first studied in the 1930s and 1940s in *Aspergillus*, *Penicillium*, and *Helminthosporium* species (Raistrick 1940; Quilico et al. 1949). Species of *Aspergillus glaucus* series was characterized by green conidial heads and hyphae with varying colors of bright yellow to red. These organisms are found as spoilage organism. Dried form of these organisms gave various pigments like auroglaucin (orange-red needles), flavoglaucin (lemon-yellow needles), and rubroglaucin (ruby-red needles) (Gould and Raistrick 1934). Studies on pigments from rubroglaucin were eventually shown to be a mixture of hydroxyanthraquinones physcion and erythroglaucin (Fig. 4.9).

4.4 Conclusion and Future Prospects

The studies discussed above reflect that the genus *Aspergillus* can be characterized with high adaptability to various ecological environments. However, it is important to mention that the results of any study aimed at the examination of *Aspergillus* biodiversity should always be evaluated in the context of the developmental stage of *Aspergillus* taxonomy and the species identification methods available at the time of the publication of the respective paper. Aspergilli have a long history in biotechnology as expression platforms for the production of food ingredients, pharmaceuticals, and enzymes. The achievements made during the last years, however, have the potential to revolutionize *Aspergillus* biotechnology and to assure *Aspergillus* a dominant place among microbial cell factories. This chapter highlighted most recent breakthroughs in fundamental and applied *Aspergillus* research with a focus on new molecular tools, techniques, and products which shed the light on an important fungus among us.

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Chapter 5
Mycorrhizal Fungi: Biodiversity, Ecological Significance, and Industrial Applications

Dheeraj Pandey, Harbans Kaur Kehri, Ifra Zoomi, Ovaid Akhtar, and Amit K. Singh

Abstract Mycorrhizae ("fungus roots") are mutualistic symbiotic associations between fungi and plants. Mycorrhizal association was found to be established between Ordovician and Devonian period. Mycorrhizal association is present in almost all ecosystems with a high degree of host specificity. About 40,000–50,000 fungal species form mycorrhizal association with nearly about 250,000 plant species. There are different types of mycorrhizal associations, namely, arbuscular mycorrhiza (71%), ectomycorrhiza (2%), orchid mycorrhiza (10%), ericoid mycorrhiza (1.4%), non-mycorrhizal association (7%), and habitat- and nutritional-dependent association (8%). These symbiotic associations play a key role in evolution of land plants in reducing and harsh environment at that time. These symbiotic associations provide up to 80% of N and P and also help in plant growth and fitness. There are a number of scientific evidences which have suggested that mycorrhizal fungi not only improve crop yield but also increase antioxidants, vitamins, and essential trace elements in plants. Additionally, various researchers around the globe have investigated the effect of mycorrhizal fungi on production of secondary metabolites. Furthermore, application of mycorrhizal fungi is presently reaching to an industrial stage supported by widespread applied researches and marketable applications emphasizing an eco-friendly and sustainable aspects.

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5.1 Introduction

Mycorrhiza (mykes, fungi; rhiza, root) is a beneficial symbiotic association between certain soil fungi and plant's inner ground parts like roots. Frank in 1885, for the first time, gave the term mycorrhiza for such association. Mycorrhizal association is present in almost all ecosystems (Read 1991; Brundrett 2009). There are four major types of mycorrhizal associations, namely, arbuscular mycorrhiza (AM), ectomycorrhiza (EcM), orchidaceous mycorrhiza (OrM), and ericoid mycorrhiza (ErM). Majority of plants have at least one type association out of four (Heijden et al. 2015). According to researchers this mycorrhizal association was established between Ordovician and Devonian period (Stubblefield et al. 1987). DNA-based phylogenetic study suggested that mycorrhiza evolved with first land plant; this evolution hypothesized for interdependency due to exchange of limiting resources (Brundrett 2002). Devonian period plant fossil rhizome of *Aglaophyton major* (400 mya) showed mycorrhiza-like structures, hyphae, and vesicles (Taylor et al. 1995). This symbiotic association plays a key role in evolution of land plants in such reducing and harsh environment at that time (Peterson et al. 1985; Helgason and Fitter 2005). The primary role of mycorrhizal fungi is to provide plant nutrients which are often in limiting conditions (Mathur et al., 1999; Clark and Zeto 2000; Augé 2001). Mycorrhizal associations with plants provides protection from soilborne plant pathogens (Newsham et al. 1995) and reduce heavy metal phytotoxicity, insect, and herbivory (Göhre and Paszkowski 2006; Bennett et al. 2009). Mycorrhization influences the organization and pattern structure of plant community in an ecosystem (Heijden et al. 2008) as well as soil microbial community in the rhizosphere (Rillig and Mummey 2006; Toljander et al. 2007). About 50,000 fungal species forms mycorrhizal associations with 250,000 plant species. These symbiotic associations provide up to 80% of nitrogen (N) and phosphorus (P) to the plants and help in their growth and fitness (Heijden et al. 2015).

In this association fungal partner acquires shelter and food from the plant and in return provides lots of benefits to the plant like better uptake of nutrients, especially phosphorus and other relatively immobile micronutrients copper, calcium, magnesium, zinc, etc., maintains water balance, alleviates metal toxicity, increases photosynthesis and hormone production, reduces oxidative stress, and provides resistance from biotic stresses. Mycorrhiza plays an important role in resisting different abiotic environmental stresses by various protective mechanisms. Several ecophysiological studies have revealed that symbiosis is important for plants to cope with stresses (Smith and Read 2008; Ruiz-Sánchez et al. 2010). Mycorrhizal fungi have adaptive homoplasticity in their physiology and metabolism which make them more tolerable and adaptive, when exposed to stress.

5.2 Types of Mycorrhizae

On the basis of hyphal penetration to the epidermis and its further development inside or outside of the host plant, mycorrhizal fungi are divided into two major types: (i) endomycorrhiza and (ii) ectomycorrhiza (Fig. 5.1). Endomycorrhiza forms arbuscules, vesicles, and spores inside the host cell that further categorized into AM, ErM, and OrM, whereas ectomycorrhiza forms Hartig net and thick mantle. There are some mycorrhizal fungi that form both types of mycorrhizal structures called ecto-endomycorrhiza. Ecto-endomycorrhiza found in members of subfamily Ericaceae and Arbutoideae (Heijden et al. 2015). Mycorrhizal infection in plants is either intracellular (endomycorrhiza) or extracellular (ectomycorrhiza), but in both conditions they penetrate the epidermis and invade cell for nutritional exchange of carbon (C), nitrogen (N), and phosphorus (P) (Smith and Read 2008). Figure 5.2 shows the root colonization process by AM fungi.

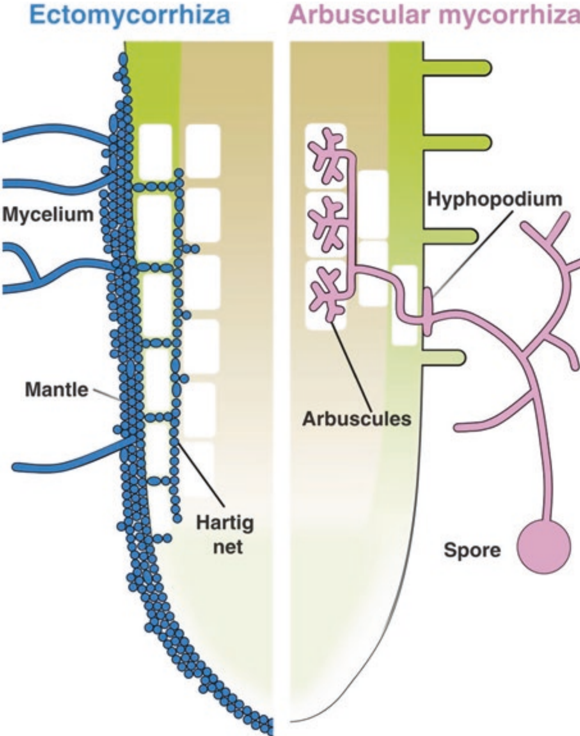

Fig. 5.1 Illustration of root colonization structures in ectomycorrhizal (blue) and arbuscular mycorrhizal (pink) interactions. (Source: Bonfante and Genre (2010))

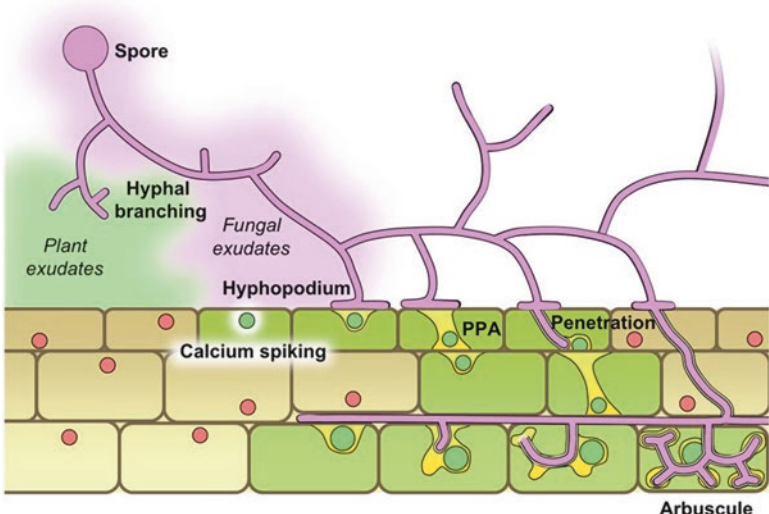

Fig. 5.2 Schematic summary of the root colonization process by AM fungi. (Source: Bonfante and Genre (2010))

5.2.1 Arbuscular Mycorrhiza

AM fungi were previously named as VAM fungi (vesicular arbuscular mycorrhizal fungi). This name is not considered in present time because many VAM fungi do not make vesicles. In present scenario more than 80% of vascular land plants and 74% of total plant species are associated with AM fungi (Smith and Read 2008; Brundrett 2009). These AM fungal associations are found in all the terrestrial ecosystems, including aquatic plants and agroecosystems as well as metal-polluted soils. But there are some families, viz., Brassicaceae, Chenopodiaceae, Polygonaceae, Juncaceae, Caryophyllaceae, and Proteaceae, which do not show mycorrhization (Smith and Read 2008). Kivlin et al. (2011) and Öpik et al. (2013) estimated 300–1600 AM fungal species belonging to phylum Glomeromycota. Out of these, AM fungi were reported to establish a symbiotic relation with the early land plants from the Devonian gametophytes (Taylor et al. 1995; Dotzler et al. 2009; Bonfante and Selosse 2010). In symbiosis AM fungi produce hyphae, arbuscules, vesicles, and spores inside the root cortex of the host and hyphae, vesicle, and spores outside the roots, but the members of family Gigasporaceae produce auxiliary cells instead of vesicles (Fig. 5.3).

The *Paris* type and the *Arum* type are two characteristic types of mycorrhiza formation with intraradial hyphal modification. *Paris* type is exclusively intracellular, forming coils in cortical cell, and found in 41 angiosperm families, whereas *Arum* type is intercellular and forms arbuscules in cortical cells and is found in 30 angiosperm families, while both types are found in 21 families (Dickson 2004; Smith and Smith 1997).

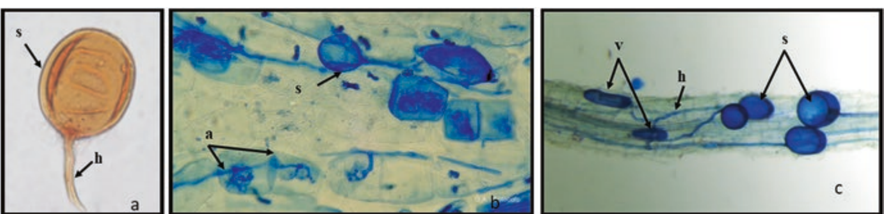

Fig. 5.3 Showing arbuscular mycorrhizal fungi: (**a**) spore of AM fungi, (**b** and **c**) AM colonization in root of the *Sorghum* plant with various structures, where h hyphae, a arbuscules, v vesicles, and s spores

Additionally, indigenous AM fungal isolates adapted to native soil types can stimulate plant growth. AM fungi can reduce negative effects of stress and serve as a filtration barrier to check transfer of heavy metals from root to the plant shoots (Schüepp et al. 1987). AM fungi are obligate biotrophs, which always need roots of living host to grow and complete their life cycles. There is no synthetic medium for proliferation of AM fungi in vitro. However, researchers have made several attempts for artificial culture media to support the growth of AM fungi (Hildebrandt et al. 2007). However, the obligate biotrophic nature of AM fungi is one of the major constrains.

5.2.2 *Ectomycorrhiza*

Ectomycorrhiza (EcM) forms important structures called Hartig net and mantle in gymnosperms, angiosperms (shrub and tree), and few liverworts of temperate region. These mycorrhizal fungi are mostly belongs from the Basidiomycota and Ascomycota (20,000 fungal species), although some members of Zygomycota also represent in this association (Rinaldi et al. 2008; Tedersoo et al. 2010). EcM symbiosis ranges over 6000 plant species (Brundrett 2009). First fossil evidence of EcM found from Jurassic period and Pinaceae might be the first plant family which makes association (Lepage et al. 1997; Hibbett and Matheny 2009). Molecular and phylogenetic evidences suggested that it originated from woody saprophytic fungal ancestors (Tedersoo et al. 2010). Limiting nutrient quantity in temperate soils of boreal forest might be the cause of EcM symbiosis (Read 1991). There are various structures formed by EcM. The Hartig net formed inside the root but mantle forms a thick condense woven structure around the root (Fig. 5.4).

Besides this some modifications also vary host to host. Hartig net is originated from the inner of the mantle and grows inside of root forming network between outer cell and root axis. This region acts as exchange site. Dense woven hyphae in mantle resemble parenchyma-like tissues referred to as pseudoparenchymatous structure (Dighton 2016). EcM suppress the development of root hairs but promote the root branching due to increased level of cytokinin in root cell (Giron et al. 2013).

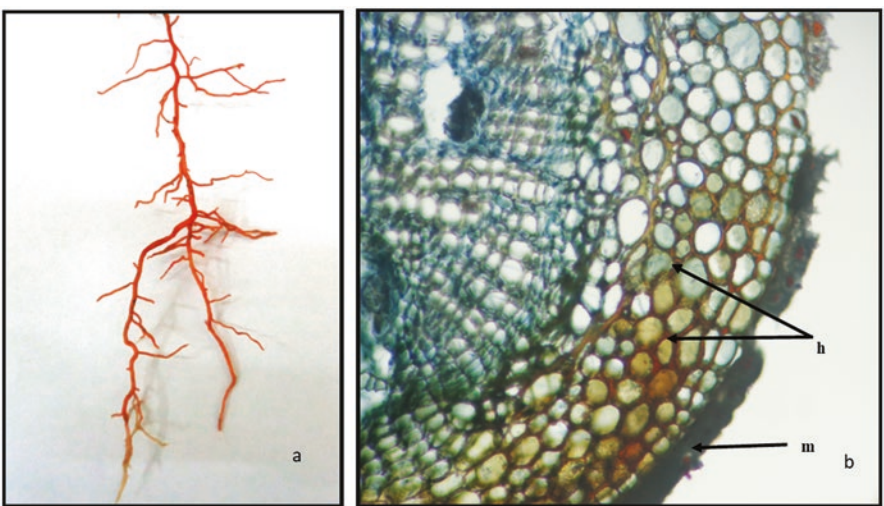

Fig. 5.4 Showing ectomycorrhizal fungi, (**a**) colonized root of *Pinus* sp. (**b**) T.S. of *Pinus* root showing h Hartig net and m mantle

Expanding of EcM hyphae out from root forms the mycorrhizal network and fruiting bodies at soil surface or above to it. These reproductive structures help in identification of EcM. EcM can accumulate high metal contents in their fruiting bodies, and that accumulation capacity varies among different species (Gast et al. 1988). Some EcM shown 60 times more Hg accumulation in comparison to soil Hg content (Bargagli and Baldi 1984).

5.2.3 Ericoid Mycorrhiza

Ericoid mycorrhizae (ErM) are found in symbiosis with members of Ericaceae and some liverworts (Read et al. 2000). Two percent of total plants are symbiotically associated with ErM (Brundrett 2009). ErM fungi mostly belong to the Ascomycota and some to Basidiomycota. More than 150 ErM species participate in symbiosis with 3900 plant species including Diapensiaceae (Walker et al. 2011; Heijden et al. 2015). Molecular and paleontological evidences suggest that ErM fungal association might have been established in age of Cretaceous period (140 mya) (Cullings 1996). ErM is well adapted in acidic and poor nutrient soil conditions. However it is not found in the subfamilies of Ericaceae like Monotropoideae, Arbutoideae, and Enkianthoideae (Heijden et al. 2015).

ErM fungi are obligately associated with living plant roots; their hyphae colonize epidermal cells separately and form characteristic fungal coils from which they reach to cortical cells and form dense interwoven hyphae. The coil is the site of exchange between host and fungi. ErM also have the capacity to degrade organic substances acting as the saprotrophs (Cairney and Burke 1998). Wurzburger et al.

(2012) support the idea of saprotroph adaptation of ErM. They investigated *Rhododendron maximum* roots' fungi by ITS (internal transcribed spacer) and rDNA methods and found 71 fungal taxa including *Rhizoscyphus ericae*, *Oidiodendron maius*, and other members of Helotiales, Chaetothyriales, and Sebacinales. Egerton-Warburton and Allen (2001) found dual symbiotic association (AM-ErM) in *Eucalyptus* and *Populus* trees. Bradley et al. (1982) investigate and find that ErM significantly decreases metal content in shoot, whereas increases in the roots of *Calluna vulgaris*, *Vaccinium macrocarpon*, and *Rhododendron ponticum*.

5.2.4 Orchid Mycorrhiza

Orchid mycorrhiza (OrM) are symbiotic association of a group of mycorrhizal fungi with plant's roots of family Orchidaceae ranging from 20,000 to 35,000 orchid species and fungal taxa about 25,000 species belonging to Basidiomycota mainly from families Tulasnellaceae and Ceratobasidiaceae but Serendipitaceae and Pezizales and multiple EcM groups also involved in orchid mycorrhization (Heijden et al. 2015). Orchid Plant's seeds are very minute spindle-shaped (0.35 mm to 1.50 mm) and with no reserve food material so OrM symbiosis is important at the time of germination of such seeds which provides carbon source for the germinating seeds. Achlorophyllous orchids will retain their fungal symbionts throughout their entire life cycle (McKendrick et al. 2002), but epiphytic orchids have chlorophyll in their leaves, stems, and roots. Orchid seed germination and development of protocorms and young plants depends on Mycorrhizal partner which promote germination of the protocorm (Alghamdi 2017). This symbiosis is obligatory for seedling development and nutrition (Rasmussen and Whigham 2002).

5.2.5 Other Categories of Mycorrhiza

Some characteristic marphological differentiation in ectomycorrhizal fungi and specialization in host plant ranges in specific plant families, there are two other subtypes of mycorrhiza:

(i) *Arbutoid mycorrhiza* – this is often the same as EcM; the only difference is the actual penetration of the cortical cell and formation of dense coiling. *Arbutoid mycorrhiza* is found in families Ericaceae and Pyrolaceae and genera *Arctostaphylos* and *Arbutus*.
(ii) *Monotropoid mycorrhiza* – this type of mycorrhizal association is found in achlorophyllous family Monotropaceae. *Monotropoid mycorrhiza* does not actually penetrate the plant cells; instead it produces fungal pegs (small protuberance) that facilitate nutrient transfer to host.

5.3 Biodiversity and Distribution of Mycorrhizal Fungi

There is total 0.5–10% (40,000–50,000) fungal species involved in mycorrhizal associations (Blackwell 2011; Taylor et al. 2014). Mycorrhizal fungi are obligate and facultative and some are also saprotrophic in nature (Heijden et al. 2015). Mycorrhizal association is well distributed in plant kingdom and increases association from lower to higher phylum. In comparison to other mycorrhiza, AM fungi showed oldest ancestral phylogeny with emergence of land plants (Selosse and Le Tacon 1998).

AM mycorrhiza makes symbiotic association with about 200,000 plant species which mostly include herb, grass, trees, hornworts, and liverworts (Brundrett 2009). AM extend passes through the different ages, faced extreme environmental conditions, and diversify in different morphotypes and similar extant mycorrhizal structures observed in plant fossils from the Triassic (Stubblefield et al. 1987). The initial period of taxonomy of AM fungi exclusively depends on few morphological characters such as sporocarp, and free, single spores are utilized in the identification and naming of AM fungi. Later the method of wet-sieving and decanting "wall layers" and other morphological parameters was adapted in the AM taxonomy. After the development of molecular techniques has opened a various new aspects in AM fungi taxonomy. Many conserved barcode regions such as SSU, ITS, LSU, mtDNA, and nrDNA have been identified and are being utilized to study of evolution and phylogeny in AM fungi taxonomy.

In phylum bryophyta 25% members are associated with AM (Davey and Currah 2006; Pressel et al. 2010). Anthocerotales members are mainly associated with AM, whereas large group with mosses (Schüßler 2000; Pressel et al. 2010; Davey and Currah 2006). Families such as Metzgeriaceae, Pleuroziaceae, Sphaerocarpales, and Ricciaceae are non-mycorrhized (Nebel et al. 2004; Ligrone et al. 2007). Sixty-seven percent of total ferns are AM associated (Zhi-wei 2000; Lehnert et al. 2017). Primitive pteridophytes, epiphytic fern, and submerged fern are mainly associated with AM, whereas leptosporangiate ferns are less dependent on mycorrhiza (Maeda 1954; Gemma et al. 1992; Nadarajah and Nawawi 1993; Lehnert et al. 2009).

AM and EcM are major mycorrhizal types, which symbiotically associated with gymnosperm. The family Pinaceae and genus *Gnetum* are symbiotically associated with EcM, whereas *Ginkgo biloba*, *Ephedra*, and *Welwitschia mirabilis* like primitive genera of this phylum are associated with AM (Maeda 1954; Jacobson et al. 1993; Titus et al. 2002; Brundrett 2002). Mycorrhizal symbiosis in flowering plants estimated 71% AM, 10% OrM, $\geq$ 2% EcM, 1.4% ErM, 7% inconsistent non-mycorrhizal AM, and remaining 8% in non-mycorrhizal (Brundrett 2017; Brundrett and Tedersoo 2018). There are some carnivores, parasites, and cluster-rooted plant species which grow in nutritional-limiting environment that are commonly non-mycorrhizal or NM-AM (Brundrett and Tedersoo 2018) (Table 5.1).

Table 5.1 Showing the percentage of plants colonized by different types of mycorrhizal fungi

Mycorrhizal fungi	Taxonomical distribution	Colonization percentage
Arbuscular mycorrhizal fungi	Mostly found in terrestrial plants	71%
Ectomycorrhizal fungi	Mostly in gymnosperms (Pinaceae and genus *Gnetum*) and Ericaceae, Pyrolaceae, and Monotropaceae, genus *Arctostaphylos*	2%
Ericoid mycorrhiza	*Calluna vulgaris*, *Vaccinium macrocarpon*, and *Rhododendron ponticum*	1.4%
Orchid mycorrhiza	Mostly found in Orchidaceae family	10%
Non-mycorrhiza	Mostly in arid, alpine, and degraded habitat	8%
NM-AM	Nutritional specialists or habitat specialists	7%

Source: Brundrett and Tedersoo (2018)

5.4 Ecological Significance

The evolution of mycorrhizal association in plants are stable, and in this association fungal partner has the ability to contribute resource exchange; response of effectiveness but it varies with plant to plant (Walter et al. 2002). In most ecosystems mycorrhizal fungi facilitate nutrient transfer, water transfer, sometimes carbon transfer, etc. In tropical ecosystem, AM fungi like *Rizophagus irregularis* and *Funeliformis mosseae* are generally present (Öpik et al. 2003, 2006). Mycorrhiza helps in seedling establishment (Heijden and Horton 2009), litter decomposition (Lindahl et al. 2007), soil formation, soil aggregation (Rillig and Mummey 2006), and plant community establishment (Yang et al. 2014) and supports plant invasion in new community (NUnez et al. 2009; Dickie et al. 2010).

5.4.1 Nutrient Cycling

In terrestrial ecosystems mycorrhizal fungi play a role in carbon, nitrogen, phosphorus, and other trace nutrient cycling. The abundance of AM fungi decreases heavy fertilization and soil disturbance. In this mycorrhizal association both partners are benefited to each other and plant might be allocate 10% to 20% of synthesized carbon resource to AM (Johnson et al. 2002; Nottingham et al. 2010), and in EcM and ErM, approximately 20% can be allocated (Hobbie and Hobbie 2008). Mycorrhiza acts as solubilizer, and its hyphal network helps in providing sufficient phosphorus to plant host in nutrient-limiting soil, and such symbiosis also regulates carbon dynamics ecosystem (Clemmensen et al. 2013). Smith and Smith (2011) reported that AM fungi contribute majority of P (up to 90%) but fewer roles in nitrogen cycling (Mäder et al. 2000; Hodge and Storer 2015). Nutrient leaching is a problem in nutrient-limiting ecosystem. In such soil mycorrhizal association binds the nutrients and reduced the leaching (Bender et al. 2015) and promotes nutrient efficiency (Fig. 5.5).

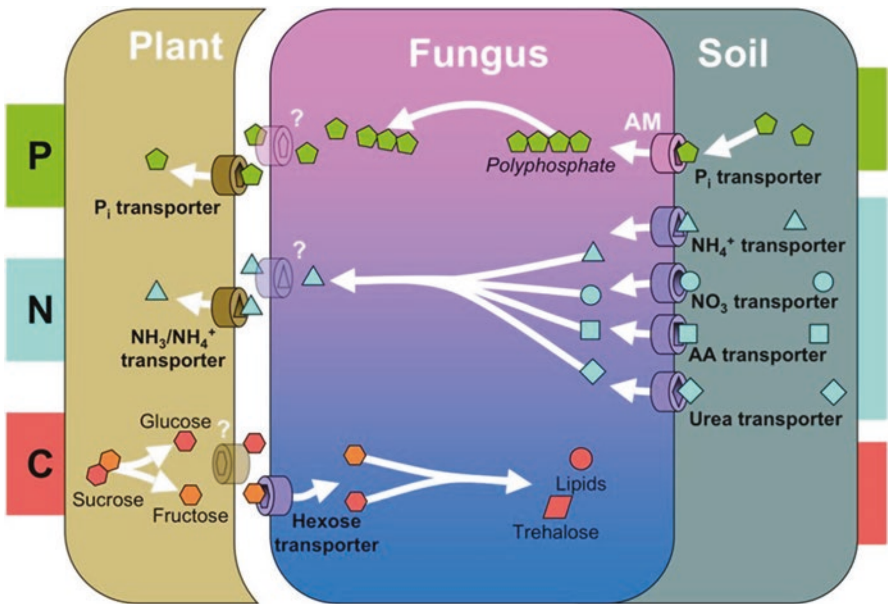

Fig. 5.5 Scheme summarizing the main nutrient exchange processes in EM and AM symbiosis. (Source: Bonfante and Genre (2010))

5.4.2 Phosphorus, Nitrogen, and Water Uptake

Nutrients are very important for plant growth and reproduction. Mycorrhizal symbiosis also facilitates mycorrhizal pathway for uptake of nutrients beyond the deficient zone of nutrients. In nutritionally poor soils, a nutrient deficient zone is created in and around rhizosphere, which is very low in nutrients. Mycorrhizal symbiosis spreads hyphae so far away from this zone and mobilizes nutrients like P, N, and other trace nutrients (Schachtman et al. 1998). P is an important nutrient for proper plant development and present in soil in form of orthophosphate, which is in immobilized form and not available for plant uptake. Mycorrhizal fungi store P in form of polyphosphate, which facilitate to keep relatively low Pi concentration in hyphae and maintain proper P transfer to plant cell (Hijikata et al. 2010) and regulate the cation homeostasis and trap heavy toxic metals and check their transport to upper ground part of plant (Bücking and Heyser 1999).

Plant P transporters are downregulated in response to AM symbiosis (Xu et al. 2007), while mycorrhizal P transporter is induced (Grunwald et al. 2009). In EcM hydrophobic protein interferes in sheath permeability for water and other nutrients (Unestam and Sun 1995). Pine trees are highly dependent on EcM symbiosis, for water and nutrient uptake (Ouahmane et al. 2009). In *Hebeloma cylindrosporum* $HcPT_1$ and $HcPT_2$ are P transporters that work in both conditions low and high P availability. AM and EcM both can take up inorganic nitrogen (N) such as ammonium or nitrate from soil (Finlay et al. 1988; Jin et al. 2005; Hawkins et al. 2000).

Some EcM are utilized nitrogen compounds as energy source and make them available for plants (Smith and Read 2008), whereas AM fungi are relatively very low to utilize organic N-sources (Hawkins et al. 2000; Jin et al. 2005; Gachomo et al. 2009). AMT 1 and AMT 2 are two ammonium transporters in *Hebeloma cylindrosporum*, involved in upregulation of nitrogen under low ammonium condition (Javelle et al. 2003). GintAMT1 is an ammonium transporter present in *Glomus intraradices* involved in ammonium transportation in low nitrogen condition (Lopez-Pedrosa et al. 2006) and absorbed ammonium and nitrate assimilated via glutamine synthase/glutamine oxoglutarate aminotransferase pathway in both AM and EcM (Jin et al. 2005; Tanaka and Yano 2005).

EcM and AM fungi play a key role in uptake water (Augé 2001). AM fungi protect host plant through drought avoidance mechanism; it maintains adequate hydration status in plant cell (Augé 2001, Augé and Moore 2005). Mycorrhizal fungi promote the accumulation of osmolytes within root cells by decreasing osmotic potential of cell for absorption of water (Serraj and Sinchair 2002; Rapparini and Peñuelas 2014), and enhancing gas exchange in plant also promotes water use efficiency in plants (Rapparini and Peñuelas 2014).

5.4.3 Amelioration of Plant Stress

Mycorrhizal symbiosis helps in amelioration of different types of plant stresses such as metal, salt, drought, and other biotic stress. All these stresses produce reactive oxygen species (ROS) that cause oxidative stress in plants. ROS negatively affect the cellular activities that cause oxidation of proteins, peroxidation of lipids, and inhibiting the enzymes activity that results in total cellular damage (Sharma et al. 2012). AM fungi enhance the antioxidant defense level in host (Wu and Zou 2009; Baslam and Goicoechea 2012; Apel and Hirt 2004) and produce isoprenoid to protect plant from several stresses (Rapparini et al. 2008).

In salt stress condition, mycorrhizal roots have a higher hydraulic conductivity at low water potential (Kapoor et al. 2008) and increase stomatal conductance, which increases the demand of transpiration (Sheng et al. 2008). In presence of mycorrhizal symbiosis, plant increases their ability to resist salt stress by accumulation of solutes and improving osmotic adjustment (Latef and Chaoxing 2014) and increases antioxidant production by increasing P uptake (Feng et al. 2002; Evelin et al. 2009) and by modification in morphology and physiology of host plant.

Heavy metal (HM) toxicity adversely affects the plant growth, development, and production. HM causes chlorosis, necrosis, senescence, turgor loss, and finally plant death. Like other stresses it also produces ROS and methylglyoxal which is involved in peroxidation of lipids, oxidation of proteins, inactivation of enzymes, and DNA damage (Candelone et al. 1995). Endomycorrhizal fungi trap HM in their binding sites and immobilize and accumulate in their mycelia. Additionally, glomalin protein produced by AM fungi provides a binding site for HM (Candelone et al. 1995).

5.4.4 Potential Application of Mycorrhizal Fungi

For the last three decades, mycorrhizal fungi have been recognized as a modern feasible biological tool in production system (Vosátka et al. 2008; Vosátka and Albrechtová 2008, 2009; Gianinazzi et al. 2010). There are a number of scientific evidences which suggested that mycorrhizal fungi not only improve crop yields but also increase antioxidants, vitamins, and essential trace elements of plants (Gianinazzi et al. 2010; Albrechtova et al. 2012). Additionally, various researchers around the globe have investigated the effect of mycorrhizal fungi on production of secondary metabolites such as terpenoids (Akiyama and Hayashi 2002), phenylpropenoid (Bruisson et al. 2016), flavonoids (Morandi 1996; Larose et al. 2002; Mechri et al. 2015), glucosinolates (Vierheilig et al. 2000; Cosme et al. 2014), stilbenoid (Bruisson et al. 2016), phenols (Zhu and Yao 2004; Hazzoumi et al. 2015), and essential oil (Kapoor et al. 2002; Kapoor et al. 2007; Hazzoumi et al. 2015). Steviol glycosides (SGs) – stevioside and rebaudioside A – enhanced in plant (*Stevia rebaudiana*) inoculated with mycorrhizal fungi (Mandal et al. 2013). In addition, indirect evidence suggests that mycorrhizal fungi also reported to affect positively the production of artemisinin (Kapoor et al. 2007; Mandal et al. 2015). On the other hand, mycorrhizal fungi can also help in decontamination of metal-polluted soils and its application into practice of phytoremediation (Vallino et al. 2006; Miransari, 2011; Bhargava et al. 2012; Meier et al. 2012). There are various reports that mycorrhizal fungi have capability to decrease the translocation of heavy metals from root to above ground part (Wu et al. 2014), thereby improving the food quality and safety (Rivera-Becerril et al. 2002; Liu et al. 2015). Thus, application of mycorrhizal fungi is presently reaching to an industrial stage supported by widespread applied research and marketable applications emphasizing an eco-friendly and sustainable aspect of the use of mycorrhiza fungi (Vosátka and Dodd 2002; Vosátka and Albrechtová 2009; Gianinazzi et al. 2010).

5.5 Conclusion and Future Prospects

In past periods of changing climatic scenario, the evolution of mycorrhizal associations coevolved with different host plants in different habitat and environmental conditions. These associations facilitate the host plant to cope with different stressed environments. Heavy metals, toxic chemicals, and water pollution make the agricultural lands polluted which decreases crop production and increases health risk. In modern agricultural practices, these irreversible changes are managed with many biological tools such as beneficial microbes. AM mycorrhiza commonly used in modern sustainable agriculture and other mycorrhizae like EcM and OrM are applied in modern forestry for seedlings preparation, establishment, and acclamation in transplantation. The key role of mycorrhiza is to maintain soil fertility and soil health and ameliorate the stresses. But in present time, decreasing plant

diversity and continuous agricultural practices adversely affect the mycorrhizal diversification. So there is the need of more knowledge about genetics, interaction biology, and tolerance and remediation mechanisms.

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Chapter 6
Fusarium: Biodiversity, Ecological Significances, and Industrial Applications

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Abstract Since Link introduced genus *Fusarium* in 1809, the genus encompasses a diverse array of species of significance for being devastating plant pathogens that often produce a wide range of secondary metabolites and attracted an immense interest. The association of some of these metabolites with cellular toxicity, effects on growth and development of animals, and cancer in humans and domesticated animals is of particular interest to agriculture and food safety. The taxonomic history of *Fusarium* species has been reviewed in great detail elsewhere. The genus currently contains nearly less than 200 accepted species, and its economic and historical importance makes it remain at center stage in future discussions about nomenclature and mycological diversity. Therefore, together with its ubiquitous nature, these species are of great significant impacts on ecosystems, agriculture, food production, biotechnology, and human and animal health. The aim of this chapter is to give an overview of the studies aimed at the investigation of *Fusarium* biodiversity in a wide variety of different ecological habitats, ecological significances, and industrial applications.

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6.1 Introduction

Fusarium is a cosmopolitan genus of filamentous ascomycete fungi (*Sordariomycetes/Hypocreales/Nectriaceae*) that includes many toxin-producing plant pathogens of agricultural importance. Collectively, *Fusarium* diseases include wilts, blights, rots, and cankers of many horticultural, field, ornamental, and forest crops in both agricultural and natural ecosystems. Members of the genus *Fusarium* are cosmopolitan and prevalent components of different ecosystems in a wide range of environmental and climatic zones, because they can colonize a wide variety of substrates. Furthermore, *Fusarium* is a typical soil-borne genus, widely distributed and generally abundant in all types of soils around the world (Backhouse et al. 2001). Some species complexes, such as the *Fusarium oxysporum* species complex (FOSC), the *Fusarium solani* species complex (FSSC), and the *Fusarium incarnatum-Fusarium equiseti* species complex (FIESC), are considered ubiquitous, while the distribution of some other species depends more on climate conditions (Summerell et al. 2010). However, several other environmental factors, including soil characteristics, crops, cultural practices, and human activities, may affect the diversity of *Fusarium* communities in soil, although the relative importance of these different parameters is poorly understood. Several soil-borne fusaria, e.g., the different formae speciales of *F. oxysporum*, which are responsible for severe vascular wilts or root rot diseases in a wide range of crops of economic importance, are important plant pathogens. The FOSC also includes several well-known biological control agents (Alabouvette et al. 2009). In addition, *Fusarium* can be found in aquatic habitats, including seawater, river water (Palmero et al. 2009), and drinking water sources (Oliveira et al. 2013), and some populations seem to be particularly adapted to complex water distribution systems (Steinberg et al. 2015). Moreover, many *Fusarium* species are of clinical importance, causing, e.g., serious corneal infections (Chang et al. 2006) and invasive infections in immunocompromised patients (Guarro 2013). Several *Fusarium* species have also been reported as pathogens of marine animals (Khoa et al. 2004, Makkonen et al. 2013).

Fusaria also produce a diverse array of toxic secondary metabolites (mycotoxins), such as trichothecenes and fumonisins, which can contaminate agricultural products, making them unsuitable for food or feed. Trichothecenes can also act as virulence factors in plant diseases (Desjardins et al. 1996; Bai et al. 2002; Desmond et al. 2008; Ilgen et al. 2008). Although opportunistic *Fusarium* infections (fusarioses) of humans and other animals are relatively rare, they typically show broad resistance to antifungal drugs (Alastruey-Izquierdo et al. 2008). Fusaria are disproportionately associated with fungal infections of the cornea (Gower et al. 2010). The genus *Fusarium* was introduced by Link in 1809 for species with fusiform, nonseptate spores borne on a stroma and was based on *Fusarium roseum*. One of the main resoans that taxonomy of *Fusarium* genus is still complex is that several species belonging to this genus charcterized by various morphological, physiological, and ecological characteristics (Edel-Hermann et al. 2015). *Fusarium* taxonomy has been plagued by changing species concepts, with as few as nine or well over a thou-

sand species being recognized by various taxonomists during the past 100 years depending on the species concept employed. The literature stabilized significantly in the early 1980s with the publications of Gerlach and Nirenberg (1982) and Nelson et al. (1983), who defined morphological species concepts that were widely accepted and successfully used by numerous practitioners. Gerlach and Nirenberg (1982) accepted 90 species based on the Berlin school (Wollenweber and Reinking 1935), while Nelson et al. (1983) accepted 43 species based on the American school (Snyder and Hansen 1940, 1941, 1945). These publications are best thought of as definitive signposts rather than as the end of the journey. Since the 1980s, the number of recognized species has increased gradually, with the number of recognized species now >80, of which 70 were described and illustrated by Leslie and Summerell (2006). The application of biological (Leslie 2001) and phylogenetic (Nirenberg and O'Donnell 1998) species concepts to the new and existing strain collections has indicated that many of the previously described species were in need of further splitting if the species designations are to be biologically meaningful. In many cases, formal descriptions of such species have been made (Klittich et al. 1997; Geiser et al. 2001; Marasas et al. 2001), or old names have been resurrected and associated with groups of strains now split from previous species (Samuels et al. 2001).

The relatively large amount of work done on the morphological taxonomy of these fungi means that as a genus *Fusarium* often has served as testing ground for new speciation concepts in fungi. The genus *Fusarium* consists of populations that are quite variable. For this reason, identification of its different species requires special culture media and methods, as well as standard incubation conditions. High variability in species, especially under different environmental conditions, has caused taxonomists to consider some special criteria to be important in the classification of species. For this reason, different methods and/or keys have been presented for the identification of the species (Booth 1975; Gerlach and Nirenberg 1982; Nelson et al. 1983; Leslie and Summerell 2006). A culture of *Fusarium* must be subcultured and purified before the identification process proceeds further. A common mistake is to try to identify the culture directly from the isolation medium. There are many isolation media for recovering *Fusarium* species such as Czapek Dox agar medium (CZDA) (Raper and Thom 1949), Peptone-PCNB medium (PPA) (Nash and Snyder 1962), Dichloran chloramphenicol peptone agar medium (DCPA) (Andrews and Pitt 1986), and Czapek iprodione dichloran agar medium (CZID) (Abildgren et al. 1987). Accurate identification of a culture requires growing it on at least two media: carnation leaf-piece agar (CLA) and potato dextrose agar (PDA) or potato sucrose agar (PSA). Carnation leaf-piece agar is a natural medium that is useful for many species of *Fusarium* which readily form sporodochia and uniform macroconidia that are particularly useful for identification purposes. PDA cultures are used primarily to assess pigmentation and gross colony morphology (Summerell et al. 2003). Also, other media used are Spezieller Nahrstoffarmer agar (SNA) (Nirenberg 1976) for producing abundant microconidia and chlamydospores (Gerlach and Nirenberg 1982), and KCL medium for the formation of microconidia in chains in section Liseola (Fisher et al. 1983).

The morphological criteria useful for identification of *Fusarium* species include two categories: primary characters which include macroconidia, microconidia, conidiogenous cells, and chlamydospores and secondary characters such as rate of growth and pigmentation. Relevant microscopic features for *Fusarium* identification include colony characteristics on either potato dextrose agar (PDA) or potato sucrose agar (PSA) (including growth rates, aerial mycelium, and colony reverse), macroconidia from sporodochia (including shape, dimensions, septation, basal cell, and apical cell), microconidia from aerial mycelium (including abundance, shape, in chains or as false heads), conidiogenous cells (mono- or polyphialidic conidiogenous cells and short or long), and chlamydospores (shape, thin or thick walled, color, and arrangement) (Booth 1971; Gerlach and Nirenberg 1982; Nelson et al. 1983; Seifert 1996; Summerell et al. 2003; Leslie and Summerell 2006). Until the 1990s, the species concept was based on morphological characters. Then, new tools like metabolite profiling and different molecular techniques came up as valuable supplements and correctives to the traditional species description (Logrieco et al. 1995a, b; Hering and Nirenberg 1995; Thrane and Hansen 1995; Gams et al. 1998, 1999; O'Donnell et al. 1998; 2000; Aoki and O'Donnell 1999; Aoki et al. 2001; Thrane 2001; Britiz et al. 2002; Dhoro 2010; Abedi-Tizaki and Sabbagh 2012).

As molecular studies progress and the definition of common and important species solidifies, the development of molecular diagnostics for many species also should be possible. These diagnostics should be much faster than the present morphological diagnosis. However, the molecular diagnostics will need to be carefully evaluated on a broad range of species as well as strains within the species to accurately define their diagnostic ability and limitations (Leslie and Summerell 2006). For laboratories that currently lack and are unable to acquire molecular expertise, morphological species definitions will remain the rule, and these laboratories should consult researchers with access to molecular technologies to confirm their identification especially for those very closely related species (Leslie and Summerell 2006).

Fusarium species are best known as plant pathogens, but a few species are commonly encountered as contaminants in food products, indoor environments, and industrial processes. Most species may be seed-borne, and many are encountered in grain (especially when it has been stored in less than optimum conditions). They may cause contamination of pharmaceutical products or machine cooling liquids. The conidia produced by most *Fusarium* species are formed in a slimy matrix facilitating dispersal by means of water rather than air. This makes *Fusarium* relatively uncommon members of the air mycoflora in comparison with *Penicillium*, *Cladosporium*, etc. *Fusarium* mycelium is transformed into the so-called mycoprotein "Quorn," a popular meat substitute acceptable to many vegetarians. Other valuable applications include the use of *Fusarium* chemicals in production of plant and animal growth promoters.

Mycotoxin production is a feature of many *Fusarium* species, including the highly toxic trichothecenes and other metabolites including zearalenone, fusarins, moniliformin, and fumonisins. Trichothecenes cause neurological disorders, immunosuppression, gastrointestinal damage, and hemorrhaging. Some species can be

implicated in infections of humans and animals and may cause problems especially for immunocompromised patients. There have been allegations of use of *Fusarium* mycotoxins as biological weapons, and at least two compounds are placed on the USDA/CDC Select Agent list and require special permission for use in research in the USA. *Fusarium* is an anamorphic genus, with teleomorph counterparts primarily in the genera *Gibberella* and *Haematonectria*. The meiotic forms are rarely encountered in culture and in many species may only be produced by crossing compatible strains in highly specific growth conditions.

Colonies usually grow rapidly and may be pale (whitish to cream) or bright colored in yellow, brownish, pink, reddish, violet, or lilac shades. Aerial mycelium may be felty, cottony, diffuse, or even absent; its production is strongly influenced by the culture medium used. Conidiophores where present are usually branched from the base, but in some species their production in culture is restricted. Often, complex pustules consisting of aggregated conidiophores (sporodochia) are formed; a confluent slimy mass of spores with a fatty or greasy appearance may be produced.

Conidiogenous cells are often slender and tapering and proliferate percurrently and usually bear a single fertile opening, though some species show several openings (so-called polyphialides). Conidia can be arranged in false slimy heads, a slimy layer over the substrate ("pionnotes"), chains, or dry masses. Two main types of conidia can be distinguished for many species, though in some morphological intermediates are formed. Macroconidia are one- to many-septate, fusiform to sickle-shaped, mostly with an elongated apical cell and pedicellate basal cell (foot-cell). Microconidia are usually one-celled and significantly smaller than macroconidia. They may be pear-shaped (pyriform), fusiform to ovoid, and straight to curved and are nearly always borne on aerial mycelium. Chlamydospores may be present or absent, occurring in intercalary or terminal positions, in solitary, or in chains or clusters. Sclerotia can be present or absent.

6.2 Diversity and Distribution of *Fusarium* Species

Fusarium species are widely distributed in all major geographic regions of the world; they are commonly found in soils and persist as chlamydospores or as hyphae in plant residues and organic matter. Many *Fusarium* species are abundant in fertile cultivated and rangeland soils, rather than in forest soils. *Fusarium* colony was found abundant and diverse in cultivated soils, and a high degree of variability in morphology and physiological characteristics enables some species such as *F. oxysporum* and *F. equiseti* to occupy the diverse ecological niches in many geographic regions (Refai et al. 2015). *F. graminearum* is the most important *Fusarium* species in central Europe and in large areas in North America and Asia. During the last years, *F. graminearum* has been spreading to the north in Europe in the Netherlands, England, Sweden, Finland, and north-western Russia, and it has been replacing the closely related *F. culmorum*, which is less effective in producing DON.

F. *graminearum* is dominant in Europe and North America. Lineage 7 of *F. graminearum* dominates in northern Europe and Asia and has been replacing the closely related *F. culmorum* in northern Europe (Refai et al. 2015).

In Iran, the diversity and prevalence of *Fusarium* species and their chemotypes on wheat in the North-West and North of Iran were determined. Wheat in these areas is severely affected by *Fusarium* head blight (FHB), with *Fusarium graminearum* as prevalent species causing 96% of the infections in the North-West and 50% in the Northern provinces. *Fusarium graminearum* strains producing 15-ADON were abundant in Ardabil (NW of Iran), while in Golestan province (N of Iran) at the other side of the Caspian Sea, especially nivalenol-producing strains and a variety of other *Fusarium* species were observed. Strains producing 3-ADON were rarely found in both areas (Refai et al. 2015). In Canada, *Fusarium* head blight of wheat and ear rot of corn causes significant yield and quality losses as well as contaminates grains with trichothecene mycotoxins. The fungus is also a potato pathogen and is routinely recovered from potato tubers showing symptoms of *Fusarium* dry rot in Canada. Interestingly, all the *G. zeae* strains from potatoes were 3-Acetyl-DON (3-ADON) types. The ability of representative isolates to produce 3-ADON and 15-ADON was verified in rice culture (Refai et al. 2015).

In Brazil, *F. graminearum* with a 15-ADON genotype is dominant in wheat (83%), followed by *F. meridionale* with a NIV genotype (12.8%), *F. cortaderiae* with mostly NIV and a few 3-acetyl deoxynivalenol (3-ADON) (2.6%), *F. austroamericanum* with mostly 3-ADON and a few NIV (1.2%), and *F. asiaticum* with the NIV genotype (0.4%). Frequency of *F. meridionale* in wheat increased with the decrease of latitudes. For the maize kernel population, *F. meridionale* is dominant (72%), followed by *F. graminearum* with the 15-ADON genotype (14.5%) and *F. cortaderiae* with the 3-ADON and NIV genotypes (13.5%). For the maize stubble population, *F. meridionale* is dominant (50%), followed by *F. graminearum* with the 15-ADON genotype (30%) and *F. cortaderiae* with the NIV and 3-ADON genotypes (20%). *F. asiaticum* with the NIV genotype is the sole species found in rice kernels. These results show that several species coexist in the subtropical to tropical agricultural regions of Brazil where host and geographic (climatic) region shape species composition (Refai et al. 2015) (Fig. 6.1).

The 3-ADON chemotype of *F. graminearum* is prevalent in Scandinavia, Finland, and north-western Russia. The 15-ADON chemotype of *F. graminearum* is more common in the more southern areas in Europe and China. Both the 3-ADON and 15-ADON chemotypes of *F. graminearum* are common in the Russian Far East (Refai et al. 2015). *Fusarium* root rot is a widespread disease of soybean in the USA and elsewhere in the world. Affecting seedlings as well as adult plants, it can be caused by numerous *Fusarium* species, and its severity is highly variable. *Fusarium oxysporum* is the most common species (Fig. 6.2), followed by *F. solani*, *F. graminearum*, and *F. acuminatum*. Representative isolates of these species cause seedling blight, root rot symptoms, and detrimental effects on root system growth and development. *F. graminearum* isolates are consistently aggressive pathogens on soybean roots. Several species are also involved such as *F. armeniacum*, *F. commune*, and *F. proliferatum*.

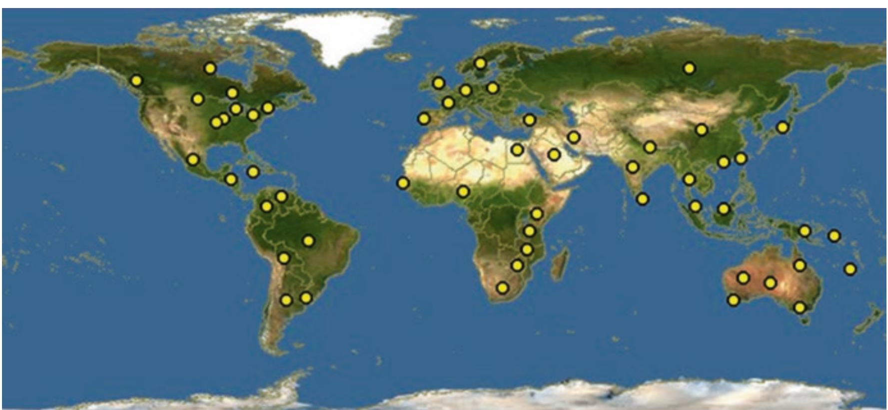

Fig. 6.1 Distribution of *Fusarium graminearum* worldwide (www.discoverlife.org)

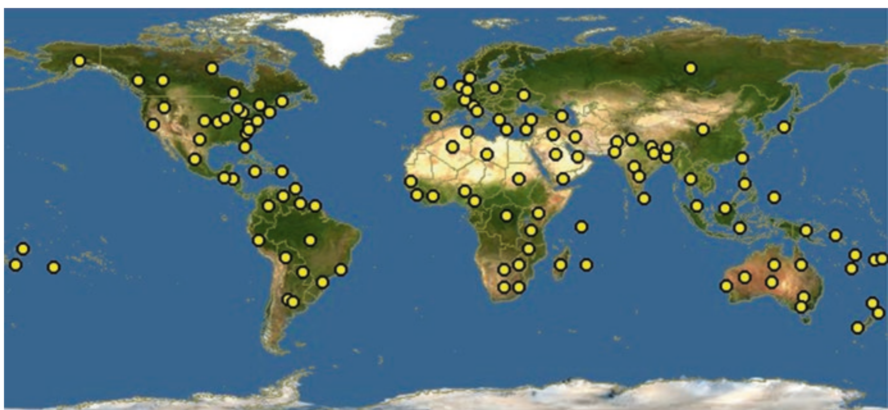

Fig. 6.2 A map depicts how *F. oxysporum* affects 6 of the 7 continents on Earth (www.discoverlife.org)

Fusarium root rot, caused by *Fusarium solani*, can cause damping-off of seedlings and root rot on older plants. Infected seedlings can result in poor weak stands, late emergence, or stunted plants. *Fusarium* root rot is an important widespread disease of field pea worldwide and can attack the crop at various growth stages, symptoms in seedlings to mature plants. *Fusarium* root rot is a problematic in Alberta since ~2010. *Fusarium* root rot is common in North Dakota, but severe damage has often been observed in association with stressed plants, such as in drought conditions or with herbicide damage. *Fusarium* root rot, or dry root rot, is the most common and important root rot of beans in North Carolina. Green bean is the main host, but lima bean, southern pea, and garden pea are also affected. It occurs mostly in hot weather in acid and poorly fertilized soils. The disease tends to be evenly distributed over a field (Refai et al. 2015).

Fusarium head blight is one of the most devastating plant diseases in the world. The US Department of Agriculture (USDA) ranks FHB as the worst plant disease to hit the USA since the rust epidemics in the 1950s. Since 1990, wheat and barley farmers in the USA have lost over $3 billion dollars due to FHB epidemics. Canada has also experienced severe losses since 1990. Major outbreaks of *Fusarium* head blight (red) on wheat and barley have been recorded by several investiigators worldwide (Refai et al. 2015). The *Fusarium* head blight-causing species are common all over Europe, but their importance is different depending on the climatic conditions. The increase in importance of *F. graminearum* reported earlier in Central Europe has been observed during the past 10 years, especially in Norway where high deoxynivalenol contents have been frequently analyzed in oats in some areas. Signs of the same development have also been observed in Sweden and Finland, where DON contaminations have previously been lower. *Fusarium* head blight species can produce mycotoxins that accumulate in the grains, creating a threat to human and animal health. In Europe, type B trichothecenes, especially deoxynivalenol (DON), are frequently found in grain batches. Most of the genes involved in producing these mycotoxins (TRI genes) are grouped in a 12-gene core cluster (TRI cluster). *Fusarium graminearum*, *F. culmorum*, and *F. cerealis* possess this cluster, but the presence or absence of certain TRI genes, as well as their functionality, results in a strain capable of producing either nivalenol (NIV) or deoxynivalenol and a related acetylated derivative (3- or 15-ADON).

Fungi of the *Fusarium oxysporum* species complex are ubiquitous soil- and plant-inhabiting microbes. As plant pathogens, *F. oxysporum* appears to be largely cosmopolitan meaning that it can be found almost everywhere, with higher concentrations of the various formae speciales in different areas across the globe. FOSC strains can cause wilt and root rot diseases on over 120 plant species. Many FOSC strains can infect plant roots without apparent effect or can even protect plants from subsequent infection. FOSC isolates also have been identified as human pathogens causing localized or disseminated infections that may become life-threatening in neutropenic individuals. *Fusarium wilt* of banana (Panama disease) is a destructive fungal disease of banana plants. It is caused by *Fusarium oxysporum* f. sp. *cubense* (Foc). It first became epidemic in Panama in 1890 and proceeded to devastate the Central American and Caribbean banana industries that were based on the "Gros Michel" (AAA) variety in the 1950s and 1960s. Once Foc is present in the soil, it cannot be eliminated. *Fusarium wilt* of banana is caused by 35 different strains or genotypes of *Fusarium oxysporum* f. sp. *cubense*. The distribution of clonal lineages of *Fusarium oxysporum* f. sp. *cubense* on banana plantations were record around the world (Refai et al. 2015).

There are four recognized races of the pathogen which are separated based on host susceptibility. Race 1, which was responsible for the epidemics in "Gros Michel" plantations, also attacks "Lady Finger" (AAB) and "Silk" (AAB) varieties. Race 2 affects cooking bananas such as "Bluggoe" (ABB). Race 3 affects *Heliconia* spp., a close relative of banana, and is not considered to be a banana pathogen. Race 4 is capable of attacking "Cavendish" (AAA) as well as the other varieties of banana

affected by races 1 and 2. These three races have been present on the east coast of Australia for many years, and race 1 is present in WA. Race 4 is further divided into "subtropical" and "tropical" strains. "Tropical" race 4 is a more virulent form of the pathogen and is capable of causing disease in "Cavendish" growing under any conditions, whereas "subtropical" race 4 generally only causes disease in plants growing suboptimally (cool temperatures, water stress, poor soil).

The strain associated with TR4 was identified in 1990 in samples from Taiwan. For the next 20 years or so, the distribution of TR4 was limited to parts of Asia and Australia's Northern Territory. The first report of TR4 outside the Asia-Pacific region dates to 2013 when it was announced that the fungal strain had been confirmed in Jordan. Later that year, it was also reported to be in Mozambique. The capacity of TR4 to survive decades in the soil, along with its lethal impact and wide host range, is among the main reasons it was ranked as the greatest threat to banana production. The severity of the damage depends on interactions between the strain, its host, and environmental conditions. To avoid further losses to the pathogen, the United Nations' Food and Agriculture Organization (FAO) has called on banana-producing countries to step up monitoring and reporting and to contain suspected incursions to prevent the fungus from getting established (Refai et al. 2015).

Fusarium wilt of tomatoes was first described by G.E. Massee in England in 1895. It is of worldwide importance where at least 32 countries had reported the disease, which is particularly severe in countries with warm climate. At one time, the disease nearly destroyed tomato production in parts of Florida and the southeastern states of the USA. However, the development and use of resistant cultivars have nearly eliminated the concern over this disease. Three physiological races of this pathogen have been reported. Race 1 is the most widely distributed and has been reported from most geographical areas. Race 2, though it was first reported in Ohio in 1940, it did not become widespread or of economic concern until its discovery in Florida in 1961. Since then, it was rapidly reported in several of the states and in several other countries, including Australia, Brazil, Great Britain, Israel, Mexico, Morocco, the Netherlands, and Iraq. Race 3 was reported in 1966 in Brazil. Thereafter, it has been found in Australia and in Florida and California. *F. oxysporum* f. sp. *lycopersici*, which causes tomato wilt, has been found in at least 32 different countries alone. *F. oxysporum* distribution maps show that this fungus has invaded North and South America, Europe, Africa, Asia, and Oceania (Refai et al. 2015).

Fusarium wilt of watermelon is one of the oldest described *Fusarium wilt* diseases and the most economically important disease of watermelon worldwide. It occurs on every continent except Antarctica, and new races of the pathogen continue to impact production in many areas around the world. Long-term survival of the pathogen in the soil and the evolution of new races make management of *Fusarium wilt* difficult (Refai et al. 2015). *Fusarium wilt* of hemp (*Fusarium oxysporum* f. sp. *cannabis*) was first described on hemp in Eastern Europe about 50 years ago but is now found throughout the Northern Hemisphere. *Fusarium wilt* of hemp is a serious disease in Eastern Europe, Italy, and Southern France. Extremely virulent strains reduce Cannabis survival by up to 80% (Refai et al. 2015).

Fusarium wilt of lettuce is of worldwide occurrence dated back to 1955 in Japan, 1990 U.S. (California; Fresno County), 1995 Iran, 1998 Taiwan, 2000 Brazil, 2001 U.S. (Arizona; Yuma County) and 2002 Italy. Races of *Fusarium oxysporum f. sp. lactucae* Races 1,2,3: Japan Race 1: Brazil, Iran, Italy, Taiwan, and the USA (Refai et al. 2015).

Fusarium wilt of cotton caused by *Fusarium oxysporum Schlechtend.* f. sp. *vasinfectum* was first identified in 1892 in cotton growing in sandy acid soils in Alabama. Although the disease was soon discovered in other major cotton-producing areas, it did not become global until the end of the next century. After its original discovery, *Fusarium wilt* of cotton was reported in Egypt (1902), India (1908), Tanzania (1954), California (1959), Sudan (1960), Israel (1970), Brazil (1978), China (1981), and Australia (1993). In addition to a worldwide distribution, *Fusarium wilt* occurs in all four of the domesticated cottons, *Gossypium arboreum* L., *G. barbadense* L., *G. herbaceum* L., and *G. hirsutum* L. Disease losses in cotton are highly variable within a country or region. In severely infested fields planted with susceptible cultivars, yield losses can be high (Refai et al. 2015).

Fusarium verticillioides is the causal agent of kernel and ear rot of maize. This destructive disease occurs virtually everywhere that maize is grown worldwide. In years with high temperatures, drought, and heavy insect damage, the disease can significantly diminish crop quality. *Fusarium verticillioides* (teleomorph *Gibberella moniliformis*) is the main fungal agent of ear and kernel rot of maize (*Zea mays* L.) worldwide. *F. verticillioides* is a highly toxigenic species since it is able to produce the carcinogenic mycotoxins fumonisins (Refai et al. 2015). The most significant economic impact of *F. verticillioides* is its ability to produce fumonisin mycotoxins. Various diseases caused by fumonisins have been reported in animals, such as liver and kidney cancer as well as neural tube defects in rodents, leukoencephalomalacia in equines, and pulmonary edema in pigs (Refai et al. 2015).

Epidemiological correlations have been established between human esophageal cancer and the consumption of fumonisin-contaminated maize in some regions of the world where maize is a dietary staple (Refai et al. 2015). *Fusarium fujikuroi* is a phytopathogenic ascomycete causing the bakanae disease ("foolish seedlings") in rice plants. This disease is triggered by the best known secondary metabolites produced by the fungus, namely, gibberellins. *F. fujikuroi* is able to produce several other well-investigated secondary metabolites which we can easily detect and quantify by now (i.e., bikaverin, fusarubin, fusarin C). *F. fujikuroi* also possesses the potential to produce a broad spectrum of further, yet unknown, secondary metabolites. A genome-wide bioinformatical screening approach revealed that the *F. fujikuroi* genome encodes 45 key enzymes for secondary metabolite production, like 18 polyketide synthases (PKSs) and 16 non-ribosomal peptide synthetases (NRPSs), all organized in putative gene clusters (Refai et al. 2015).

Fusarium avenaceum is often associated with diseased grains in temperate areas, either alone or in co-occurrence with other *Fusarium* species, but its prevalence is also increasing in warmer regions throughout the world. The major problems caused by *F. avenaceum* are crown rot and head blight of wheat and barley and the contamination of grains with mycotoxins (Refai et al. 2015). In Finland and other northern

agricultural areas, *F. avenaceum* is a common fungus on living and dead organic substrates. It is frequently found on cereal grains, where it may cause seedling and head blight and produce mycotoxins. *F. avenaceum* is associated with foot and root rot diseases of all cereals grown in Finland. A wide range of variation in pathogenicity between isolates has been reported (Refai et al. 2015).

In Norway, *Fusarium avenaceum*, *F. graminearum*, *F. culmorum*, *F. langsethiae*, and *F. poae* are some of the most common fungal species causing *Fusarium* head blight in cereals. *F. graminearum* has shown increased prevalence the last decade, resulting in increased deoxynivalenol contamination of cereal grains. The increased prevalence of *F. graminearum* in Norwegian cereals is likely to be associated with the recent increased use of reduced tillage in combination with weather conditions promoting development and dispersal of this fungal species (Refai et al. 2015). *Fusarium proliferatum* is considered worldwide as an emerging pathogen of garlic. *F. proliferatum* is known to produce fumonisins B1 and B2 on different vegetable matrices, and fumonisin contamination of garlic bulbs has been already reported in Germany (Refai et al. 2015). *Fusarium langsethiae* is a new European species of type A trichothecene producer. *F. langsethiae* can be divided into two lineages based on molecular markers. The European *F. langsethiae* has only been found in Europe, while the Asian *F. langsethiae* in Siberia and the Russian Far East seems actually to be a lineage of *F. sporotrichioides* based on molecular data (Refai et al. 2015).

In Finland, increase of *F. langsethiae*, the most important producer of T-2 and HT-2 toxins, has already been observed on oats and barley under reduced tillage. While DON production is enhanced by high humidity, *F. langsethiae* can infect and produce toxins in dry conditions (Refai et al. 2015). *F. sibiricum* is distributed in Siberia and Russian Far East with two single isolates from Norway and Iran. So, it is probable that the actual distribution of *F. sibiricum* will be much larger than the present known distribution (Refai et al. 2015). *Fusarium temperatum* is a new described species occurring on maize in Belgium, closely related to *F. subglutinans*. Both species are considered morphologically identical and associated to the *Fusarium* maize ear rot disease complex (Refai et al. 2015).

6.2.1 Fusarium in Egypt

In Egypt, *Fusarium* has received considerable attention from the pathological viewpoint (Abd-El-Aziz 1970; Abdel-Fattah 1973; Ashour et al. 1973; Abd-Elkader et al. 1978; Ahmed 1978; Aly 1978; Rushdi et al. 1980a, b; Mohamed et al. 1981; Arafa et al. 1986; Shihata and Gad El-Hak 1989; Abdel-Kader and Ashour 1999; El-Mohamedy 2004; El-Mohamedy et al. 2006; El-Bramawy 2006; El-Bramawy and Shaban 2007; Osama 2007; Sallam and Abdel-Monaim 2012; Ziedan et al. 2012), but its ecology has not received much consideration. Only two Ph.D. theses presented by Abdel-Hafez (1981) and Nafady (2008), on the genus *Fusarium* from Egyptian cultivated, desert, and salt marsh soils as well as seasonally fluctuated in cultivated soil and air, were conducted (Mazen et al. 1982, 1991,

Moubasher et al. 1984). Moubasher (1993) in his textbook on soil fungi in Qatar and other Arab countries made an excellent contribution of the genus *Fusarium* and its telemorphs with 14 species being well illustrated, described, and given their ecological distribution.

6.2.1.1 Terricolous *Fusarium* of Egypt

Moubasher and Moustafa (1970) found that *Fusarium* was the third commonest fungus in Egyptian soils after *Aspergillus* and *Penicillium*. It was represented by four species, namely, *F. moniliforme*, *F. oxysporum*, *F. semitectum*, and *F. solani*. Moubasher and Abdel-Hafez (1978a) found also that *Fusarium* ranked third according to the number of cases of isolation from Egyptian agricultural soils. Five species were collected, and these were *F. oxysporum*, *F. moniliforme*, *F. solani*, *F. concolor*, and *F. equiseti* which comprised 0.48%, 0.6%, 0.54%, 0.05%, and 0.01% of total fungi, respectively. Abdel-Fattah et al. (1977a) isolated three species of *Fusarium* from Egyptian salt marsh soils, and these were *F. oxysporum*, *F. moniliforme*, and *F. solani*. They occurred in 44.6%, 12.2%, and 7% of the samples, contributing 1.1%, 0.5%, and 0.7% of total fungi, respectively.

Bagy (1979) isolated six species of *Fusarium* from Egyptian soils, and these were *F. acuminatum*, *F. culmorum*, *F. equiseti*, *F. moniliforme*, *F. oxysporum*, and *F. solani*. Maghazy (1979) isolated three species of *Fusarium* (*F. moniliforme*, *F. oxysporum*, and *F. solani*) from soil treated with keratinaceous material. Moubasher et al. (1990) found that *Fusarium* was recovered very frequently from cultivated, desert, and saline soils on 5% NaCl-Czapek agar, but it was isolated with low or rare frequency on 10% NaCl-Czapek agar. It was encountered in 68%, 64%, and 56% of the samples constituting 7.9%, 4.3%, and 29.1% of total fungi in the three soil types on medium supplemented with 5% NaCl, respectively. From the genus, eight species were collected, and the most common were *F. solani* and *F. oxysporum* in cultivated and desert soils. *F. equiseti* was isolated in moderate frequency from saline soils, but it was of rare frequency in the other two types of soils. *F. graminearum*, *F. lateritium*, *F. moniliforme*, *F. poae*, and *F. roseum* were less frequently recovered (Moubasher et al. 1990).

Mazen et al. (1991) identified seven species in addition to two varieties of the genus *Fusarium*. Of these species, *F. solani* was the most frequent followed by *F. oxysporum*, *F. equiseti*, *F. acuminatum*, and *F. semitectum*; *F. moniliforme* and *F. sulphureum* were recovered in low frequency, while *F. sambucinum* var. *coeuleum* and *F. moniliforme* var. *subglutinans* were rarely isolated. Based on his comprehensive reviewing of soil fungi in Egypt, Moubasher (1993) stated that *Fusarium* was more frequently isolated in agricultural than in salt marsh and reclaimed soils. Abdel-Hafez (2004) isolated three species of *Fusarium* from newly reclaimed soil (Petroleum's farm) at Assiut Governorate, of which *F. oxysporum* and *F. solani* were the most common. On the other hand, Seddek (2007) identified five species of the genus *Fusarium*, of which *F. verticillioides* was the most common followed by *F. culmorum*, *F. oxysporum*, *F. dimerum*, and *F. acuminatum*.

6.2.1.2 Monthly Fluctuations of *Fusarium* in Soil of Egypt

The term monthly fluctuation means studying composition, numbers, and incidences of soil fungi during the different months of year which is expected to change according to the wide change in the climatic factors. Monthly fluctuation of soil fungi has been studied by several workers (Warcup 1957; Witkamp 1960; Reddy 1962; Fincher 1963; Suprum 1963; Gams and Domsch 1969; Fathi et al. 1975; El-Abyad and Ismail 1976; Ali et al. 1977; Moubasher et al. 1988; Abdel-Hafez et al. 1989).

In Egypt, Moubasher and El-Dohlob (1970) and Moubasher and Abdel-Hafez (1978b) found that the monthly counts of *Fusarium* in cultivated soils from Assiut Governorate seasonally fluctuated giving peaks during autumn or winter and minimum in summer. Mazen and Shaban (1983) found that the highest periods in soil fungi in wheat field at El-Minya Governorate were recorded during May 1977 and 1978. Also, the periods of February 1978 and December and April 1977 showed fairly high fungal population. *Fusarium* was the most frequently encountered species after *Aspergillus*. Abdel-Hafez et al. (1989) found that *F. solani* was irregularly fluctuated in soils of Wadi Qena at eastern desert during the periods from January to December 1985. *F. oxysporum*, *F. acuminatum*, *F. verticillioides*, *F. equiseti*, and *F. graminearum* were isolated, but with different counts and incidences, from non-rhizosphere soil of sugarcane filed in Qena Governorate on glucose, cellulose, and Czapek's agar media, and their maxima were recovered during various months as reported by Abdel-Hafez et al. (1995). Gherbawy et al. (2006) reported that *Fusarium* species rarely appeared at the beginning of the season and increased sharply between January and March and decreased slightly or sharply at the end of the season according to the type of media and isolation source. They isolated 14 *Fusarium* species from wheat field, of which *F. merismoides*, *F. oxysporum*, and *F. sambucinum* were the most common followed by *F. anthophilum*, *F. aquaeductuum*, *F. chlamydosporum*, *F. dimerum*, *F. moniliforme*, *F. poae*, *F. proliferatum*, *F. scirpi*, *F. solani*, *F. sporotrichioides*, and *F. subglutinans*.

6.2.1.3 Airborne *Fusarium* in Egypt

Air is seldom free from fungal spores, and the cosmopolitan distribution of fungi has been attributed to the fact that fungi occupy microenvironments which occur in various ecosystems and geographical areas (Richards 1956; Gregory 1973; Lacey 1975; Moubasher 1993). Air is one of the main sources of contamination, and several microorganisms are present in the air due to numerous causes such as animal and human activities, dust, and aerosols produced by solid waste and waste treatment facilities and by talking, coughing, or sneezing (Lighthart and Frisch 1976; Graham 1980), and several of these organisms are well-known to be pathogenic to plants, animals, and humans (Frey et al. 1979; Sehgal et al. 1981; Rippon 1982; Treger et al. 1985; Velez and Diaz 1985; Arianayagam et al. 1986; Chabasse et al. 1989; de Hoog et al. 2000).

In Egypt, knowledge on the seasonal variations of airborne fungi was focused on the air of some cities or fields at Delta area and Upper Egypt (Saad 1958; Ali et al. 1973; Abu El-Souod 1974; Moubasher and Moustafa 1974; Moubasher et al. 1981, 1982; Mazen and Shaban 1983; Youssef and Karam El-Din 1988; Abdel-Hafez et al. 1990b, 1993; Ismail et al. 2002). Abu El-Souod (1974) in her survey of airborne fungi at Assiut reported that *Fusarium* emerged in 77 and 74 daily exposures out of 366 at low and high levels, respectively. The genus *Fusarium* ranked eighth and ninth in the order of total counts (0.7% at every level) at low and high levels, respectively. The highest monthly record at low level was made during December when it was isolated in 16 days, but at high level, it was made during November (20 days). Moubasher and Moustafa (1974) reported that *Fusarium* ranked ninth in total count (1.1% of total fungi) and in frequency of occurrence (33 exposures out of 54). They identified three species of *Fusarium*, namely, *F. moniliforme*, *F. oxysporum*, and *F. semitectum*, which comprised 0.93%, 0.09%, and 0.06% of total fungi, respectively. Mazen et al. (1982) in their study on the seasonal fluctuation of airborne fungi at Assiut, Egypt, isolated 41 species belonging to 20 fungal genera, of which *Aspergillus*, *Alternaria*, and *Cladosporium* were the most common followed by *Curvularia*, *Penicillium*, and *Epicoccum*. On the other hand, *Fusarium* occupied the seventh place according to their number of cases of isolation. Only five *Fusarium* species were identified, of which *F. moniliforme* and *F. oxysporum* were the most common followed by *F. solani*, *F. equiseti*, and *F. sulphureum*.

Moubasher et al. (1988) studied the seasonal fluctuations of airborne fungi of Wadi Bir-El-Ain at eastern desert during the period from March 1978 to February 1980. They found that the monthly counts of airborne fungi seasonally fluctuated giving peak during autumn.

Twelve fungal species were frequently isolated, of which *Fusarium* was isolated in high frequency of occurrence. Abdel-Hafez et al. (1989) isolated *F. equiseti* and *F. solani* from one exposure each (out of 36 exposures) in the atmosphere of Wadi Qena during the period January-December 1985.

On the other hand, Abdel-Hafez et al. (1993) found that the genus *Fusarium* was irregularly fluctuated in the outdoor air at Assiut over a period of 2 years during January-December 1985 and 1986. Of the genus, four species were identified, and these were *F. equiseti*, *F. moniliforme*, *F. solani*, and *F. xylarioides*. Their maxima were recorded at various months.

El-Said and Abdel-Hafez (1995) studied the seasonal variation of airborne fungi above banana fields in Qena, Upper Egypt, and found that *Fusarium* was recovered in moderate frequency of occurrence on plates of glucose- and cellulose-Czapek's agar at 28 °C and the maximum was recorded during November 1992. From the genus 10 species were collected and the most common were *F. oxysporum* and *F. verticillioides*. The remaining species were recovered in low (*F. acuminatum*, *F. equiseti*, and *F. graminearum*) or in rare (*F. nivale*, *F. poae*, *F. semitectum*, *F. tricinctum*, and *F. avenaceum*) frequency of occurrence. Omar et al. (1996) found that *Fusarium* occupied the third place after *Aspergillus* and *Penicillium* in the outdoor and indoor atmosphere of Ismailia city during the period from March 1992 to May 1993. The maximum counts of *Fusarium* were estimated in March and either

September and October. Also, the prevalence of airborne mycobiota at six different regions of western desert and eastern desert of Egypt was determined using the exposed plate method by Ismail et al. (2002), and six species were encountered, namely, *F. dimerum*, *F. oxysporum*, *F. acuminatum*, *F. verticillioides*, *F. solani*, and *F. equiseti*.

6.3 Ecological Distribution of *Fusarium*

6.3.1 Rhizosphere and Rhizoplane Fusarium

Because of the widespread interest in the parasitic fungi attacking roots, numerous investigations have been made to characterize the fungus flora of root surface (Katznelson et al. 1948; Davey and Papavzas 1960; Srivastava and Mishra 1971; Foster 1986; Campbell and Neher 1996). Successful manipulation of rhizosphere and rhizoplane microorganisms to enhance biological disease control depends on knowledge of their ecological associations (Schroth and Hancock 1981; Mandeel and Baker 1991). The previous investigations achieved by Moubasher and his co-workers presented a good evidence that *Fusarium* is one of the basic constituents of fungi in the rhizosphere and rhizoplane of many Egyptian plants (Abdel-Fattah et al. 1977b; Moubasher and Abdel-Hafez 1978a, b; El-Hissy et al. 1980; Moubasher et al. 1984; Mazen et al. 1982, 1991; Moubasher 1993; Abdel-Hafez et al. 1990a, 1995, 2009; Hasan 2002; Abd-Elhafez 2004; Gherbawy et al. 2006; Seddek 2007; Ismail et al. 2009).

Abdel-Hafez (1974) recovered five species of *Fusarium* from the rhizosphere of cotton seedlings (*F. oxysporum*, *F. moniliforme*, *F. solani*, and *F. semitectum* and *F. equiseti*) and three species from rhizoplane (*F. oxysporum*, *F. moniliforme*, and *F. solani*). Also, *F. oxysporum*, *F. moniliforme*, and *F. solani* were recovered, but with different incidences, from rhizoplane of broad bean (Abdel-Fattah et al. 1977b) and rhizoplane and rhizosphere of cotton seedlings (Abdel-Kader et al. 1978). El-Hissy et al. (1980) reported that *Fusarium* was frequently recovered from the rhizosphere of five plants, namely, *Helianthus annuus*, *Chrysanthemum coronarium*, *Nigella sativa*, *Datura innoxia*, and *Hyoscymaus muticus*, in Egypt. Three species were identified, and these were *F. moniliforme*, *F. oxysporum*, and *F. solani*. Moubasher et al. (1984) isolated five *Fusarium* species in the rhizoplane of healthy and damped-off cotton, pea, tomato, maize, and wheat seedlings raised in the field during 12-month experiment, of these *F. solani* and *F. oxysporum* were the most common species followed by *F. moniliforme*, *F. acuminatum*, and *F. equiseti*. However, maize roots were surpassed by *F. moniliforme*, which was very scarce in the roots of the other test plants.

Abdel-Hafez et al. (1990b) found that *Fusarium* was one of the commonest fungi in the rhizosphere and rhizoplane of wheat plants cultivated in El-Minya Governorate and the most species were *F. oxysporum* and *F. semitectum* or *F. solani* based on the

examined source. Abdel-Hafez et al. (1995) studied seasonal fluctuation of rhizosphere soils and rhizoplane fungi of sugarcane during the periods from January to December 1992 using glucose-, cellulose-, and 50% sucrose-Czapek's agar media at 28 °C. *F. oxysporum*, *F. poae*, *F. sambucinum*, *F. acuminatum*, *F. verticillioides*, and *F. equiseti* were isolated from rhizosphere, while *F. dimerum*, *F. oxysporum*, *F. poae*, *F. verticillioides*, *F. equiseti*, and *F. sambucinum* were isolated from rhizoplane of sugarcane plants. On the other hand, Abdel-Hafez et al. (2000) isolated *F. oxysporum*, *F. verticillioides*, and *F. solani* from the rhizosphere of wheat fields in El-Kharga Oasis. Hasan (2002) isolated 14 species belonging to 7 genera from rhizosphere and rhizoplane of faba bean, melochia, sesame, and soya bean. *Fusarium* was represented only by *F. oxysporum*. Abd-Elhafez (2004) studied the monthly fluctuations of rhizosphere and rhizoplane fungi of some cultivated plants in newly reclaimed areas of Wadi El-Assiuty, Assiut Governorate, during the periods from October 2001 to September 2002. The counts of *Fusarium* in the above two habitats were irregularly fluctuated giving maxima on November and April, respectively. Six species of *Fusarium* were identified, and these were *F. culmorum*, *F. equiseti*, *F. moniliforme* var. *subglutinans*, *F. oxysporum*, *F. semitectum*, and *F. solani*.

In a study of fusaria and other fungal taxa associated with rhizosphere and rhizoplane of lentil and sesame at different growth stages, Abdel-Hafez et al. (2012) isolated 16 species of *Fusarium* from rhizosphere (13 species) and rhizoplane (11) of both plants studied. In lentil, 11 species were recorded from its rhizosphere (9 species) and rhizoplane (8). *Fusarium* species associated with lentil rhizoplane gave the highest number of propagules at the first stage of plant growth, while the ones of *Fusarium* associated with the rhizosphere produced the highest number at the second stage of growth. *F. solani* was the most common in the three growth stages. In addition, of two growth stages, *F. culmorum* and *F. tricinctum* were isolated from the rhizosphere while *F. nygamai* and *F. verticillioides* from the rhizoplane. The other species were recorded from only one growth stage of lentil plant. In sesame plants, rhizosphere yielded nine *Fusarium* species, while rhizoplane gave only six from the three stages investigated. Stage I of sesame rhizosphere possessed the highest colony-forming units of *Fusarium*. As the case for lentil, *F. solani* was the most common species in sesame rhizosphere and rhizoplane. *F. verticillioides* and *F. nygamai* (in three different growth stages) followed by *F. oxysporum* and *F. tricinctum* (in two growth stages) were recorded using the dilution-plate and/or soil-plate methods from sesame rhizosphere soils. Rhizoplane *Fusarium* species of sesame plants were isolated at the three different growth stages with almost equal number of colony-forming units. *F. poae* came after *F. solani* in its frequency since it was recovered from two growth stages. Several of the isolated species are well-known as pathogens to many cultivated plants (Abdel-Hafez et al. 2012).

It was found that several of the isolated *Fusarium* species are well-known as pathogenic to numerous cultivated plants in Egypt (Abdel-Razik et al. 1976; Hussein et al. 1977; Abdel-Kader et al. 1978; Higgy et al. 1978; Rushdi et al. 1980a, b, 1981; Mohamed et al. 1981, 1982; Ziedan 1993, 1998; Ziedan et al. 2012; Khalifa 1997;

Sahab et al. 2001; El-Mohamedy 2004; El-Mohamedy et al. 2006; Morsy 2005; El-Bramawy 2006; El-Bramawy and Shaban 2007; El-Bramawy and Abdel-Wahid 2007, 2009; Sallam and Abdel-Monaim 2012).

6.3.2 Grain/Seed-Borne Fusarium Species

Fungi carried on or within grain or seed can reduce grain or seed germination or seedling emergence (Neergaard 1977). Some plant pathogenic fungi kill seedlings shortly after they emerge, whereas others cause serious disease epidemics after being transmitted from grain/seed to seedlings. Determining what proportion (incidence) of seeds in a given seed lot are contaminated by a fungus (either externally or internally) is therefore of interest to plant disease epidemiologists (Maude 1996; Agarwal and Sinclair 1997). Gilbert et al. (1997) reported that use of the infected seed/grain without treatment results in lower plant densities.

The natural contamination of seeds with seed-borne fungi plays a vital role in determination of seed quality (Abdel-Monem 2000). Sesame (*Sesamum indicum* L.) seed is an important oilseed widely grown and used in some African and Asiatic countries. It is an important source of protein in the developing countries, and the name Benniseed is used throughout West Africa (Felixtina 1988). Sesame oil is mainly utilized as a salad and cooking oil or in the manufacturing of margarine. Lentil (*Lens esculenta* Medic.) seed is one of the oldest known protein-rich food legumes (Stoilova and Pereira 1999). Lentil wilt, caused by *Fusarium oxysporum* f. sp. *lentis*, is one of the main limiting factors to successful cultivation (Stoilova and Chavdarov 2006). It is an important and widely distributed legume crop grown under a broad range of climates (Abdel-Hafez 1988; El-Nagerabi and Elshafie 2000).

Moubasher et al. (1979) identified *F. oxysporum*, *F. moniliforme*, *F. solani*, and *F. equiseti* in peanut seeds and shells. *F. oxysporum* was the most common. On the other hand, 32 species belonging to 17 genera were recovered from lentil seeds, of which *Fusarium* species (*F. moniliforme*, *F. solani*, *F. semitectum*, *F. equiseti*, *F. oxysporum*, and *F. roseum*) were isolated in high frequency of occurrence (Abd-Allah and Hashem 2006). Embaby and Abdel-Galil (2006) found that *Fusarium* was the common species isolated from some legume (bean, cowpea, and lupine), emerging in 5.6%, 4.4%, and 4.4% of total fungi, respectively. *F. oxysporum* was the most common species.

Maize (*Zea mays* L.) grain is one of the most important dietary staple foods in the world (FAO 2002). Maize plays an important role in the diet of millions of African people due to its high yields per hectare, ease of cultivation and adaptability to different agro-ecological zones, versatile food uses, and storage characteristics (Asiedu 1989). In Egypt, maize is one of the most important and essential crops, especially in upper Egypt, not only as food for animal and human but also for Egyptian economics because the crop is used mainly in several food industries

(Abdel-Hafez et al. 2003). Several fungi are associated with maize during pre- and post-harvest periods, of which the genus *Fusarium* contains important toxigenic species (Fandohan et al. 2005). These include *F. verticillioides* which is one of the most economically important species worldwide (Shephard et al. 1996; Munkvold and Desjardins 1997; Marasas 2001; Taligoola et al. 2004). Many studies have been conducted in several parts of the world to evaluate the natural occurrence of *Fusarium* in maize (Shephard et al. 1996; Marasas 2001; Ismail et al. 2003). Kossou and Aho (1993) reported that fungi could cause about 50–80% of damage on farmers' maize during the storage period if conditions are favorable for development.

Sorghum (*Sorghum durrum* L.) is the fourth most important cereal in Egypt (after maize, wheat, and rice) and is the only one of these cereals that can be easily cultivated in the "new lands" or in very hot and arid Upper Egypt. *Fusarium* species in the *G. fujikuroi* species complex are widely known from maize and sorghum in Egypt. A common perception is that Fusarium species in the *G. fujikuroi* species cause stalk; ear and kernel rot and produce mycotoxins such as fumonisins and moniliformin. Moubasher et al. (1972), Abdel-Kader et al. (1979), Abdel-Hafez and Abdel-Kader (1980), El-Kady et al. (1982), Abdel-Hafez et al. (1987, 1992), Abdel-Mallek et al. (1993), El-Maghraby et al. (1995), and Abdel-Sater et al. (1995) isolated 13 species of *Fusarium*, but with different counts and incidences from some Egyptian cereals grains, and these were *F. oxysporum*, *F. moniliforme* (= *F. verticillioides*), *F. solani*, *F. equiseti*, *F. acuminatum*, *F. semitectum*, *F. poae*, *F. decemcellulare*, *F. tabacinum*, *F. dimerum*, *F. moniliforme* var. *anthophilum*, *F. subglutinans*, and *F. sambucinum*. Aziz et al. (2007) found that *Fusarium* infection of wheat, maize, and barley grains ranged from 25% to 40%, 30% to 60%, and 10% to 25%, respectively. Five species of *Fusarium* were collected, and the most common species was *F. moniliforme* (38.6% of total *Fusarium*) followed by *F. proliferatum* (29%), *F. graminearum* (16.5%), *F. subglutinans* (9.1%), and *F. oxysporum* (6.8%).

In 2008, Nafady was possible to characterize and identify 820 isolates into 34 species of *Fusarium* belonging to 8 sections: section Arthrosporiella (3 species, 45 isolates), section Discolor (5, 38), section Elegans (1, 91), section Gibbosum (5, 43), section Lateritium (4, 93), section Liseola (10, 189), section Martiella (1, 202), and section Sporotrichiella (5, 119) in Egypt. High proportion of the isolates was identified as *Fusarium solani* (202 isolates); the other species such as *F. oxysporum* (91), *F. verticillioides* (80), *F. nygamai* (62), *F. udum* (42), *F. lateritium* (39), *F. chlamydosporum* (35), *F. sporotrichioides* (29), *F. semitectum* (27), *F. poae* (25), *F. equiseti* (24), *F. subglutinans* (21), and *F. tricinctum* (21) came after *F. solani* in their numbers. The other 21 species were represented all by 120 isolates. She recorded seven species as new records to Egypt, namely, *F. acutatum*, *F. longipes*, *F. nisikadoi*, *F. nygamai*, *F. pseudoanthophilum*, *F. pseudonygamai*, and *F. thapsinum* (Figs. 6.3 and 6.4).

6.4 *Fusarium*'s Bioactive Metabolites

6.4.1 Antioxidants

With today's interest in new renewable sources of natural antioxidants, microorganisms are considered a potential source of isolation. Small size, simple nutrient requirements, and short life span made them widely varied habitats than any other

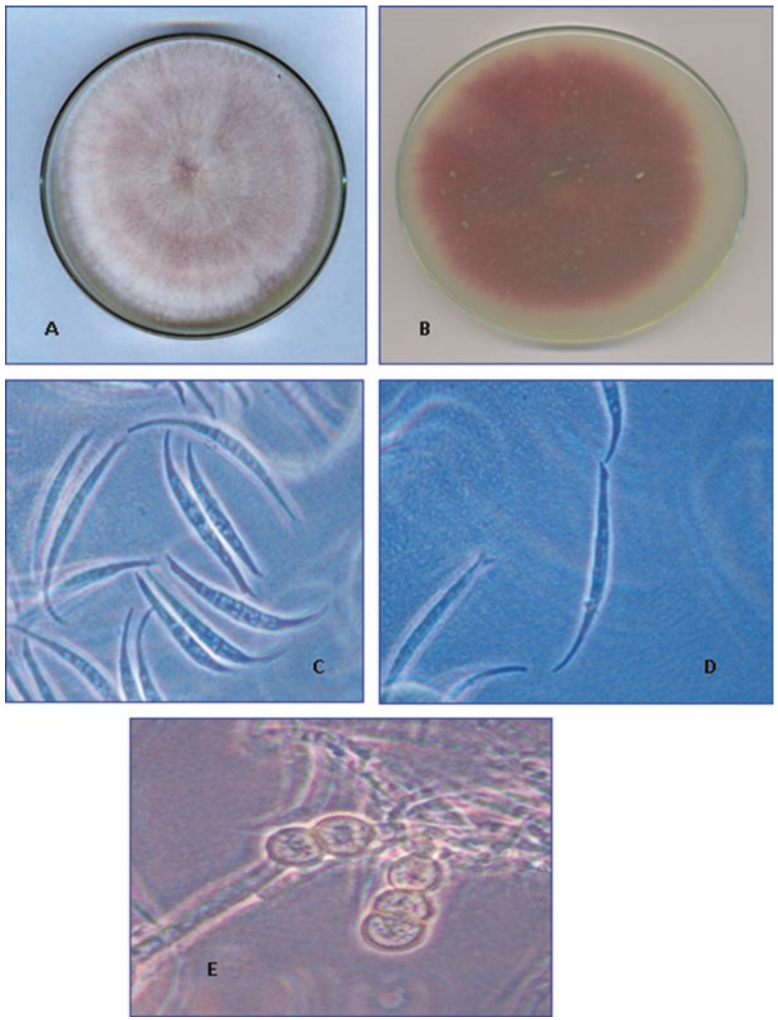

Fig. 6.3 *Fusarium longipes* Wollenweber and Reinking, (**a**, **b**): colony color and reverse on PSA. (**a**–**e**): Photographs. (**c**, **d**): Macroconidia. (**e**): Chlamydospores (© Nieven A. Nafady). *Fusarium longipes* Wollweber and Reinking, (**f**–**i**): S.E.M. (**f**): Sporodochia. (**g**, **h**): Macroconidia. (**i**): Chlamydospores. (© Nieven A. Nafady)

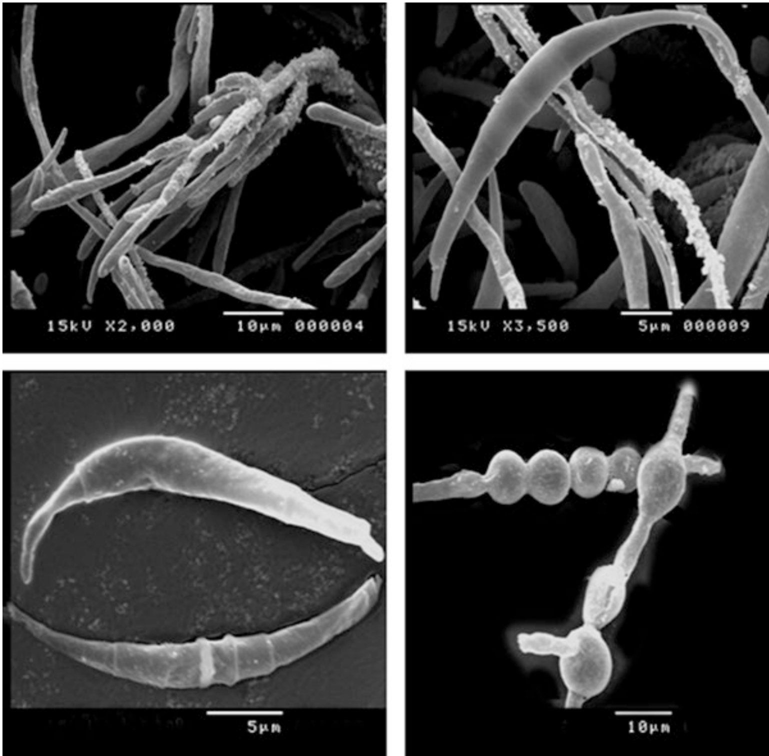

Fig. 6.3 (continued)

organisms. In recent years, new natural antioxidant molecules have gained a great importance in science and medicine. Among microorganisms, fungi have been recognized as the most fruitful sources to produce a varied range of secondary bioactive metabolites such as antimicrobial, antioxidant, antitoxic, and anticancer compounds with the various types of pharmaceutical and therapeutic applications (Rana et al. 2016a, b, 2017; Salehi et al. 2016; Yen and Lee 1996). A considerable number of fungi including higher basidiomycetes, lower filamentous fungi, and yeasts from different ecological niches were known for their ability to synthesize EPSs in laboratory culture systems. However, many still remain uninvestigated or under explored. Endophytic fungi are such type of organisms that resides in the plant tissues without apparent harm to their host and have got special interest as they are biologically rich source of centuple active substances (Yang et al. 2018). *Fusarium* species is widely distributed in various habitats which were used for exopolysaccharide (EPS) production (Li et al. 2014).

On the basis of the number of sugar units, carbohydrates are classified into three groups: monosaccharides, oligosaccharides, and polysaccharides. The natural macromolecules composed of several monosaccharide units (more than ten) are known as polysaccharides and are synthesized at different stages of life cycle of every living

organisms for different purposes. The monosaccharide units of polysaccharides are joined to each other by acetal linkages which formed by the reaction of a hemiacetal hydroxyl group of one unit with an alcohol group of another unit which liberates water to give a glycosidic bond. Polysaccharides not only have different sequences of monomeric units but also have different sequences of glycosidic linkages and different types of branching (Mahapatra and Banerjee 2013a). Fungal polysaccharides are well-known for the medicinal properties such as antioxidant, anti-inflammatory, antitumor, antiaging, antiviral, antiulcer, neuroprotective, and immunological

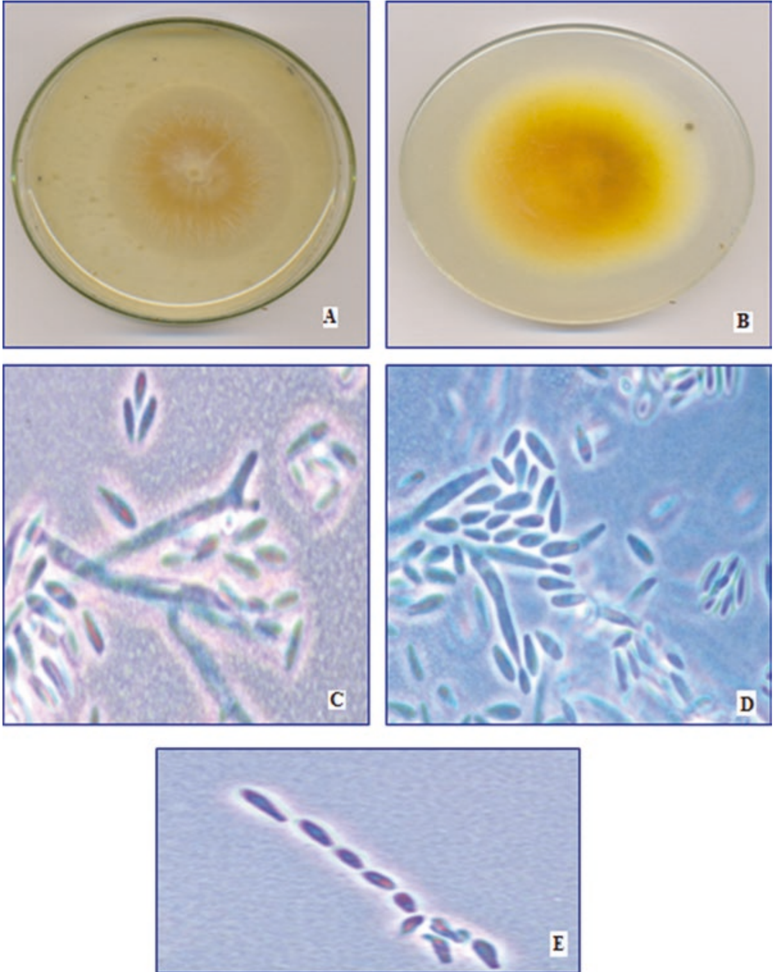

Fig. 6.4 *Fusarium nisikadoi* T. Aoki and Nirenberg, (**a**, **b**): Colony color and reverse on PSA. (**a**–**e**): Photographs. (**c**, **d**): Poly- and monophialidic conidiogenous cells. (**e**): Microconidia in chains (© Nieven A. Nafady). *Fusarium longipes* Wollweber and Reinking, (**f**–**i**): S.E.M. (**f**): Sporodochia. (**g**, **h**): Macroconidia. (**i**): Chlamydospores. (© Nieven A. Nafady)

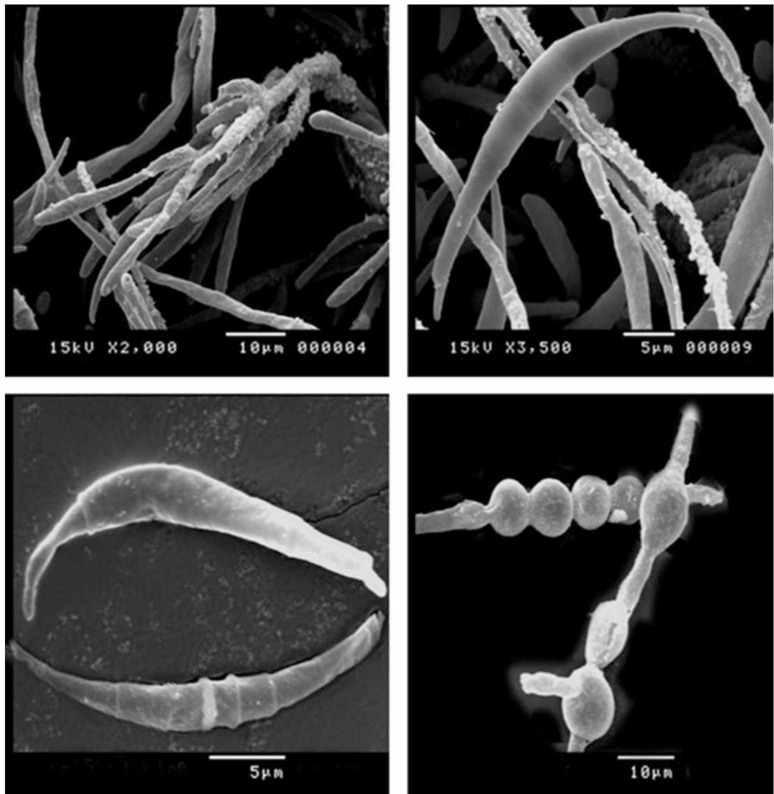

Fig. 6.4 (continued)

activities. So, novel fungi that have capability to synthesize unique polysaccharide with antioxidant properties became the scientists' target, and multiple new methods have been used for the extraction of polysaccharides including enzyme-assisted extraction (EAE), microwave-assisted extraction (MAE), and ultrasonic-assisted extraction (UAE) (Zhu et al. 2016). Carbohydrate antioxidants are expected to have better applicability as they are easily isolated and purified and water soluble and have less chances of toxicity toward cell. Moreover, various fungi-originated products have been demonstrated to trigger defense mechanisms by inducing defensive responses leading to plant defense enzyme activity enhancement, especially carbohydrate compounds (i.e., polysaccharides and oligosaccharides).

Fungal EPSs have several applications in the food, feed, cosmetic, medicine, and pharmaceutical industries. The activities of fungal carbohydrate compounds are dependent on different content and arrangements during polymerization of its building unit: monosaccharides. Their composition varies from pure sugars to sugars combined with a second unit such as protein, phosphate, sulfate, or amine. Different types of sugar unites were found in fungal EPSs such as glucose, mannose, galactose, xylose, fucose, and rhamnose. It was also noticed that EPSs composed of the

same monosaccharide units that were synthesized by different fungi had different molecular weight. This is caused by differing chain length or branching patterns that give polysaccharides a great diversity of structure, property, and functions. For instance, the extracellular polysaccharide produced by the mangrove-associated fungus *Fusarium oxysporum* Dzf17 is defined as galactofuranose-containing mannoglucogalactan, consisted of galactose, glucose, and mannose in a molar ratio of 1.33:1.33:1.00, and its molecular weight was about 61.2 kDa. The structure contains (1 → 6)-linked β-D-galactofuranose, α-D-glucopyranose, (1 → 2)-linked α-D-glucopyranose, (1 → 2)-linked β-D-mannopyranose, and β-D-mannopyranose. Its EPS showed elicitor activities on growth and diosgenin production in cell suspension culture of *Dioscorea zingiberensis* (Chen et al. 2015). EPS of endophytic *Fusarium solani* SD5 is characterized as heteropolysaccharide of galactose and rhamnose, rhamno-galactan, containing a hexasaccharide repeating unit with a novel structure with molecular weight 1.87 × 105 Da. The structure contains α-L-rhamnopyranosyl, (1 → 2)-α-L-rhamnopyranosyl, (1 → 4)-β-D-galactopyranosyl, and (1 → 4,6)-β-D-galactopyranosyl moieties in a molar ratio of nearly 1:1:3:1. This EPS has significant mast cell stabilizing and membrane protective activities. Thus, this exopolysaccharide may offer significant effects for preclusion of inflammatory and allergic conditions in vitro. In addition, it is evident as a nontoxic, free radical scavenger and a good antioxidant which nominate it as a promising health-boosting drug (Mahapatra and Banerjee 2012, 2013b). However, the chemical characterizations of the oligosaccharides (purification of oligosaccharide monomers, monosaccharide composition, monosaccharide linkage of each oligosaccharide monomer) as well as their structure-activity relationships and more specific defensive mechanisms are not clear and worth investigating.

6.4.2 Enzymes

Enzymes can be currently used in several areas, and the main applications include removal of fat, starch, and protein stains in tissues using lipases, amylases, and proteases. In dairy products, proteases and lipases are used in milk coagulation and cheese ripening; in baking, amylase is applied in dough strengthening, while pectinases and cellulases are applied in beverages, for juice depectinization and fruit liquefaction, respectively. Furthermore, xylanases are extensively applied in animal feed to enhance starch digestibility, while in pulp and paper industry, delignification and deinking are enhanced by the use of laccase and cellulase; and in biofuel production, the use of cellulases and amylases is crucial for cellulose hydrolysis (Kirk et al. 2002; Singh et al. 2016; Sahay et al. 2017; Suman et al. 2015; Yadav et al. 2016).

Recently, some studies have highlighted enzyme production from *Fusarium* species for biotechnological purposes (Table 6.1). Mechanisms involving experimental design and optimization of process parameters are performed in order to obtain a maximum enzyme activity (Yusuf et al. 2013; De Castro and Sato 2013; Almeida et al. 2014; Soni et al. 2016). Additionally, the use of agricultural wastes as substrates

Table 6.1 Recent enzymes from *Fusarium* strains and main mechanisms and applications

Enzyme	Producer strain	Mechanisms and/or applications	References
Laccase	*F. incarnatum* LD-3; *F. solani* MAS2	Pulp and paper industries, drug analysis, wine clarification as well as biotransformation of environmental pollutants, also in nanobiotechnology for the development of biosensors to detect various phenoloic compounds, oxygen, or azides	Chhaya and Gupte (2013), Wu and Nian (2014)
Chitinase	*F. oxysporum* CFR 8	Reclamation of seafood processing crustacean by-products, production of bioactive N-acetyl chitooligosaccharides, and production of N-acetyl-D-glucosamine	Thadathil et al. (2014)
Protease	*F. oxysporum*	Protein hydrolysis used in various industries	Ali and Vidhale (2013), Deshmukh and Vidhale (2015)
Chitin deacetylase	*F. oxysporum*	Conversion of chitin to chitosan by the deacetylation of N-acetyl-D-glucosamine units	Suresh et al. (2014)
β-glucosidase	*F. oxysporum*	Hydrolyses cellobiose by cleaving the β-(1–4) linkage in it to generate D-glucose, used in fuel industry	Olajuyigbe et al. (2016)
Lipase	*Fusarium* sp.; F. *heterosporum* expressed in *Aspergillus oryzae*	Hydrolyses ester bonds in triglycerides, promoting the production of biodiesel with the advantage of increasing reaction rates and removing lipid strains from tissues	Oliveira et al. (2013) and Amoah et al. (2016)
β-Mannanase	Various *Fusarium* strains	Participation on mannan hydrolysis, releasing manno-oligosaccharides	Soni et al. (2016)
Nitrilase	*F. proliferatum* (AUF-2)	Catalyze hydrolysis of the triple bond of the cyano group of nitriles to form corresponding carboxylic acids with the removal of nitrogen as ammonia	Yusuf et al. (2013)
Endoglucanase and xylanase	*F. verticillioides*	Hydrolysis of cellulose and hemicellulose for biofuel production	Almeida et al. (2014)
Cellulase	*F. oxysporum* F3; *F. solani*; *F. subglutinans* MTCC 11891	Lignocellulosic biomass hydrolysis for biofuel production	Xiros et al. (2011), Panagiotou et al. (2011), Behera and Ray (2016), and Indira et al. (2016)

and the fermentation system performed (submerged or solid state) are also reported as important tools in the search for optimal production processes. Wu and Nian (2014) investigated the potential production of the enzyme laccase (p-diphenol: dioxygen oxidoreductase) by the *F. solani* MAS2 strain. For this purpose, the

authors used an experimental study with a mineral salt medium under liquid fermentation, involving response surface methodology (RSM), to verify the effects of the parameters temperature, pH, and anthracene concentration and the carbon source used.

Maximum amount produced (159.78 U/mL laccase) was achieved at 20 °C and pH 6.5 with 30 mg/L initial anthracene concentration, reaching a 38.9-fold production relative to nonoptimized conditions. Chhaya and Gupte (2013) studied the laccase production potential by *F. incarnatum* LD-3 under solid-state fermentation. Using the "one factor at time" approach, maximum laccase production was achieved at pH 5.0 and 28 °C, with a substrate containing 60% of rice bran. After supplementation with 2% (v/v) of alcohol, laccase production increased 52.56%, reaching 1352.64 U/mg under optimized conditions, 2.1-fold higher than previously established conditions. Thadathil et al. (2014) employed commercial wheat bran for the thermoactive chitinase production in solid-state fermentation using *F. oxysporum* CFR 8 and response surface methodology to evaluate the process parameters. Using incubation temperature, incubation time, initial moisture substrate content, and inoculum (spore suspension) concentration as independent variables, the results showed an endo-chitinase activity of 17.5 U/g of initial dry substrate and 319.9 U/g of initial dry substrate of β-N-acetylhexosaminidase, with optimum crude extract activity at 62 °C temperature in a wide pH range. Therefore, this fungus is a potential commercial producer of extracellular chitinase.

Fusarium strains have also been extensively studied for the production of proteases, important enzymes commonly obtained from animal and plant sources. Ali and Vidhale (2013) evaluated the effect of incubation time, initial moisture content, incubation temperature, and initial pH value for the protease production by *F. oxysporum* under solid-state fermentation, using rice bran obtained from rice mill. Maximum protease activity found was 70.5 U/g after 72 h of incubation, with an initial moisture content of 50% (w/w) at 35 °C and pH 7.0, proving that the rice bran can be an economically suitable substrate. In another study, protease production by *F. oxysporum* was investigated through the use of various agro-industrial wastes (dal mill waste, oil mill waste, molasses, waste fruit, and vegetable garbage), in order to evaluate the effect of pH on each of these substrates. Using solid-state fermentation, researchers obtained maximal protease production (21.8 μg/mL) at pH 5 after 120 h of incubation using vegetable garbage as substrate, showing the high potential of this agro-industrial waste for protease production (Deshmukh and Vidhale 2015).

Currently, some companies are employing proteases and alpha-amylases from *Fusarium* sp. for cleaning purposes. The patent previously assigned to Novo Nordisk (US5288627A, nowadays assigned to Novozymes) claims the application of endoproteases produced by *F. oxysporum* DSM2672 with specific characteristics for use in detergent formulations (Nielsen et al. 1994). Regarding alpha-amylase properties in increase the removal of starchy stains during laundry washing or dishwashing, Procter and Gamble Company (PandG) developed cleaning products compositions comprising variants of this enzyme (Jackson et al. 2013).

The enzyme chitin deacetylase has its activity reported by Suresh et al. (2014) in several fungal species. In this approach, these authors studied chitin deacetylase production in solid-state fermentation by native soil isolate *F. oxysporum*, using by-products of fresh marine shrimp processing and commercial wheat bran as substrate. After substrate preparation and spore inoculation at 32 °C followed by chilled extraction, the results show maximum chitin deacetylase activity 306.4 U/g dry substrate after 72 h incubation in wheat bran medium and 220.1 U/g dry substrate after 120 h incubation in shrimp by-product medium. Besides that, *F. oxysporum* also produced other chitin-degrading enzymes such as endo-chitinase and β-N-acetylhexosaminidase, achieving 7.8 U/g dry substrate with maximum endo-chitinase activity after 144 h incubation and 38.3 U/g dry substrate β-N-acetylhexosaminidase activity after 120 h incubation in wheat bran medium.

Olajuyigbe et al. (2016) studied the production of the β-glucosidase during methyl cellulose biodegradation by *F. oxysporum*. The researchers used medium containing methyl cellulose, KH2PO4, ZnSO4.7H2O, FeSO4.7H2O, MnSO4.7H2O, MgSO4.7H2O, CaCl2, CoCl2, (NH4)2SO4, yeast extract, urea, and peptone at pH 6.0 and incubated for 192 h under submerged fermentation. Best results were obtained after 96 h of fermentation with the highest concentration of thermostable β-glucosidase (177.5 U/mg) at pH 6.0 and 30 °C and liberation of 2.121 μmol/mL glucose, showing that the enzyme has technological feasibility and can be used on an industrial scale for cellulose hydrolysis.

In order to evaluate the application of agro-industrial wastes as substrate in lipase production by *Fusarium* sp. (*Gibberella fujikuroi* complex) isolate FCLA-MA-41, Oliveira et al. (2013) compared and optimized submerged and solid-state fermentation using a central composite design. After optimization, in submerged fermentation, average composition resulted in lipase cost reduction of 72% and 80% on Triton X-100 and yeast extract, respectively, with lipase activity of 3 U/mL. According to the authors, solid-state fermentation was the most economic bioprocess for producing lipases at a cost of US$ 28 per million units of lipase using crambe meal and water.

A Brazilian group reported the use of *F. oxysporum* 152b strain, isolated from Brazilian Northeast fruits (Maceió, AL, and Aracaju, SE) in biotechnological production of alkaline lipase (Prazeres 2006). The enzyme was characterized and evaluated to verify its stability, optimum pH and temperature, as well as the effect of surfactants and detergents on the enzyme activity. The results showed that best conditions were pH 8 and 50 °C, being that the stability of the enzyme remained 93% of residual activity during 1 h of incubation at 60 °C. Moreover, its compatibility with ionic and nonionic surfactants was described, suggesting that this lipase can be used as potential additive in detergent formulation (Prazeres et al. 2006). In this perspective, PandG patented (WO2013116261 A2) the use of microbial lipases, including enzymes from *Fusarium* sp., in methods and compositions for treating textile and hard surfaces (Lant et al. 2013). Lipases, as well as cutinases, have also been used in other cleaning agents' composition, acting as lipolytic enzymes for the removal of fatty acid-based dirt and stains (Andre and Charmoille 1999).

For evaluation of biological activity, the alkaline lipase from *F. oxysporum* 152b was mixed with a biosurfactant produced by *Bacillus subtilis* (AL/BS), and it evaluated the antimicrobial effect on several types of microorganisms, according to the minimum inhibitory concentration (MIC). It was proved that the mixture AL/BS affected the growth of *B. subtilis* CCT 2576, *B. cereus* ATCC 10876, and *Listeria innocua* (Quadros et al. 2011).

The same researcher group proposed the co-production of enzymes and aroma compounds using the *F. oxysporum* 152b strain (Bicas et al. 2010a). Authors used the biomass obtained after lipase production procedure as a 72-h inoculum for terpenes biotransformation. As the lipase production takes about 72 h to reach the final enzyme concentration (14 U/mL) and both processes occur from different pathways, the residual biomass could be reused for aroma production. Although promising, this co-production process needs to be more studied since the concentration of α-terpineol obtained was 50% lower when compared to the conventional process. Soni et al. (2016) carried out a screening of several *Fusarium* strains with potential production of enzyme β-mannanase (β-1,4-mannan mannohydrolase) which the main function is hydrolysis of polymers with manno-oligosaccharide release, required in fuel production. Researchers conducted fermentation tests using several inducers for enzyme production, such as locust gum, bean gum, guar gum, or konjac gum in a basal medium, incubated at 28 °C for 4 days (submerged fermentation) or substrates like wheat bran, wheat straw, rice husk, fenugreek seed meal, and *Aloe vera* pulp supplemented with copra meal, palm kernel cake, locust bean gum, guar gum, konjac gum, glucose, mannose, and solka floc as inducers, incubated at 28 °C for 6 days (solid-state fermentation). Also, two parameters, pH and moisture content, were selected as variables in a central composite design in order to optimize β-mannanase production. Results showed maximum β-mannanase by *F. equiseti* (1747 nkat/gds), selected for statistical optimization on palm kernel cake, which resulted in three- to fourfold enhancement in enzyme yield (5945 nkat/gds).

Optimization of nitrilase production (nitrile aminohydrolases, EC 3.5.5.1) was reported by Yusuf et al. (2013). After the isolation of the strain *F. proliferatum* AUF-2 as a nitrilase producer, several parameters (incubation temperature, pH, percentage of inoculum, agitation, carbon and nitrogen source, and inducers) were evaluated for maximum enzyme production in submerged fermentation. The optimized conditions were pH 7.0, 200 rpm, 28 °C, and 72 h of fermentation, leading to a twofold (2000 U/L) increase in nitrilase production when compared to previous activity. Considering the above, it is worth noting that enzyme production obtained from fungal *Fusarium* species can be a technical and economical feasible process, since it makes use of techniques such as submerged or solid-state fermentation in addition to process optimization tools. Studies also emphasize the importance of research and use of low-cost agro-industrial wastes as alternative substrates, aiming the development of sustainable and environmental friendly processes.

6.4.3 Biofuels

Biofuels can be described as solid, liquid, or gaseous fuels from biological feedstocks (biomass), for example, biochar, bioethanol, biobutanol, biodiesel, biohydrogen, and biomethane, which can be used to replace petroleum-derived fuels (Harish et al. 2015). Among the biofuels, bioethanol can be highlighted due its feasibility to be used as transportation fuels and as substrate in some chemical reactions, for example, alcoholysis in biodiesel production. Moreover, it can be produced from different kinds of feedstocks, such as sugarcane, corn, wheat, cassava, cellulosic biomass, and algal biomass (Baeyens et al. 2015). The first-generation biofuels are produced from sugarcane, sugar beet, and corn starch (bioethanol) and also from palm tree, an oleaginous plant for biodiesel production. Although most of the biofuels used nowadays are obtained from these sources, they are often considered as unsustainable, unable to meet the global demand of bioethanol, and its production is limited by the competition with food production (Vohra et al. 2014; Harish et al. 2015; Gupta and Verma 2015).

Second-generation biofuels are produced from non-edible lignocellulosic biomass that includes crop residues, grasses, sawdust, and other agro-processing by-products. It is interesting to use this kind of biomass for fuel production because it is an abundant source of sugars for fermentation and their availability does not impact in land use (Vohra et al. 2014; Menon and Rao 2012). The main differences between first- and second-generation biofuels are the steps of pretreatment and hydrolysis of lignocellulosic sugars required before fermentation (Brown 2015). The biomass needs to be processed in order to release fermentable hexoses and pentoses that can be metabolized by fuel-producers microorganisms, and those pretreatments may also produce inhibitors that prejudice fermentation performance or the concentration of sugars obtained after hydrolysis are not sufficiently high to product formation (Vohra et al. 2014; Lennartsson et al. 2014). Due to the advantages of second-generation biofuel production, this process has been widely studied mainly focusing on pretreatment and hydrolysis of biomass, hydrolyzate detoxification, and also improvement and genetic engineering of strains for fermentation (Aditiya et al. 2016; Zabed et al. 2016; Paulova et al. 2015; Alfenore and Molina-Jouve 2016; Maitan-Alfenas et al. 2015; Nigam and Singh 2011; Yang et al. 2015; Mäkelä et al. 2014).

A variety of microorganisms have the ability to secrete the enzymes endoglucanases, exoglucanases or cellobiohydrolase, and β-glucosidase that are necessary for lignocellulosic biomass hydrolysis, among them fungal strains such as *Trichoderma* sp., *Penicillium* sp., *Phanerochaete* sp., *Humicola* sp., *Schizophillum* sp., and *Fusarium* sp. (Gupta and Verma 2015). In a recent review, Behera and Ray (2016) comprise relevant information on cellulase production by a variety of microorganisms (bacteria, fungi, and ascomycetes) in solid-state fermentation. It discussed the potential application of this fermentation system as well as methods and strategies to improve enzyme yields, such as metabolic engineering and genetic modification of strains, optimization of growth conditions, and design/modeling of bioreactors.

Among the fungi described in this work, *F. solani* has been cultivated in solid-state fermentation, where only a small amount of basal mineral salt medium is added to substrate. In this case, the use of solid-state fermentation is more advantageous than submerged fermentation due to the high product stability, lower catabolic repression, and reduced costs.

A strain of *F. oxysporum* F3 isolated from cumin has been extensively studied for cellulase and xylanase production in both solid-state and submerged fermentation for bioethanol production. The studies began in 1989 with the strain isolation and identification of its ability to ferment glucose, xylose, cellobiose, and cellulose directly to ethanol (Christakopoulos et al. 1989). Since then, strategies to improve enzymes activities and bioethanol yields from different carbon sources have been evaluated (Panagiotou et al. 2003, 2005; Xiros et al. 2008, 2009; Xiros and Christakopoulos 2009). More recently, alkali treated brewers spent grain (ABSG) was used to study cellulose and hemicellulose hydrolysis by an enzyme extract of *F. oxysporum* (Panagiotou et al. 2011; Anasontzis and Christakopoulos 2014; Gupta and Verma 2015). For the first time, a mathematical model expressing the factors affecting ABSG hydrolysis could successfully described and predicted sugars release during the process (Xiros et al. 2011).

Panagiotou et al. (2011) used a mixed culture of *F. oxysporum* et al. 2016; Anasontzis and Christakopoulos 2014; Gupta and Verma 2015). The use of ionic liquids (IL) in the pretreatment of lignocellulosic material was reported, making the substrate more prone to cellulose degradation. The use of IL is advantageous since they are very effective and environmentally friendly and can be recycled in different rounds of pretreatment. However, the presence of residual IL can decrease cellulase activity and inhibit microbial fermentation. Therefore, Xu et al. (2015) isolated the strain *F. oxysporum* BN from chemical-polluted microhabitats, able to grow in 10% (w/v) of 1-ethyl-3- methylimidazolium phosphinate. The cellulase produced by this microorganism also proved to maintain its activity in the presence of ILs below a concentration of 10%, being the highest resistance associated to ILs based on phosphate and sulfate radicals. In a CBP experiment, using rice straw as carbon source (1–10% w/v), *F. oxysporum* BN was able to produce bioethanol with a final yield of 64.2% (0.125 g ethanol/g rice straw). Considering the feasibility of using IL for pretreatment and the needs to obtain resistant cellulose producers strains, there are still few reports on this subject.

Another interesting renewable fuel is biodiesel, which is usually produced through alcoholysis of vegetable oils and animal fats. Nowadays, there is an increase in the researches focusing in the use of microbial lipases as whole-cell catalysts for biodiesel production (Amoah et al. 2016; Koda et al. 2010). *F. heterosporum* is a recognized lipase-producing strain; therefore, many studies have been using this fungus or the expression of the lipase-encoding gene in different host microorganisms.

Koda et al. (2010) used a recombinant strain of *Aspergillus oryzae* expressing *F. heterosporum* lipase (r-FHL) as catalyst in ethanolysis of rapeseed oil. The results indicate that r-FHL is a promising catalyst, with yields for fatty acid ethyl ester (FAEE) of 94% and the possibility of recycling the cells in repeated reactions with

no decrease in enzyme activity. It was also shown that only a small amount of water is required in the process; therefore, a water-containing bioethanol can be directly utilized, without the need of additional water. In the first report using *A. oryzae* expressing *F. heterosporum* lipase in immobilized system, it used methanol for the production of fatty acid methyl esters (FAME). In this study, it obtained 94% of FAME after 10 batch cycles, and the addition of 5% water in the reaction mixture was necessary to prevent lipase inactivation by methanol (Hama et al. 2008).

Although most of the studies uses methanol for alcoholysis, its substitution for ethanol is advantageous, since it is less toxic and it is a renewable material that can be produced from various kinds of biomass. Amoah et al. (2016) used the same recombinant strain (FHL) in a study to optimize biodiesel production from high-phospholipid content oil. It was found that decrease in agitation and higher water concentration lead to improvement of threefold in conversion efficiency and final biodiesel production of 90% from a microbial oil containing about 30% of phospholipids. This occurs because agitation causes the formation of phospholipid-based reverse micelles, in which water molecules can be trapped, resulting in the inactivation of lipase enzyme; therefore, a simple technique such as reducing agitation and increase in the water content may be successfully implemented to overcome challenges in biodiesel production.

Due to the increasing demand for renewable sources for energy production, new methods for biofuel production or strategies to increase its concentration are required. *Fusarium* sp. strains proved to be an interesting biocatalyst to be used for both second-generation ethanol and biodiesel production due to their feasibility to produce cellulases and lipases used in these processes and their ability to utilize different sugars for growth and ethanol fermentation. Therefore, studies in these areas should be encouraged in order to obtain a process that may be cost-attractive and competitive industrially.

6.4.4 Bioflavors

Flavors and fragrances have a wide application in the food, feed, cosmetic, chemical, and pharmaceutical sectors (Vandamme 2003). These compounds influence greatly the flavor of food products and govern their acceptance by consumer (Bicas et al. 2010b). In this sense, the increasing consumer preference for natural products has encouraged remarkable efforts toward the development of biotechnological processes for the production of flavor compounds (Krings and Berger 1998; Bicas et al. 2009). The microbial production of these compounds is based on two different approaches, including de novo synthesis (fermentation) or bioconversion of natural precursors with microbial cells or enzymes (biotransformation) (Bicas et al. 2009).

In general, microorganisms are capable to produce an amazingly broad array of flavor compounds by de novo synthesis. Several fungal strains are related to the production of natural flavor compounds, such as floral flavors by *Ceratocystis* sp. and *Trichoderma viride* and mainly due to the large biodiversity that occurs

especially in the *Ascomycetes* and *Basidiomycetes* orders (Vandamme 2003; Feron and Waché 2006). Few descriptions in the technical literature present developments using *Fusarium* strains as the main biocatalyst of the fermentative system through de novo synthesis. Former results showed the production of a lactone with a peach-like aroma, identified as cis-6-dodecen-4-olide (2 mg/L), when *F. poae* was grown on a solid malt medium until sporulation (Sarris and Latrasse 1985). This product was naturally found in peach, mushroom and dairy products; it is applied mainly in baked goods and milk products and has a threshold value lower than 0.5 ppm (Burdock 2010).

The production of natural 2-heptanone was described using *F. poae* as biocatalyst. Process was carried out in a 3-L fermenter and variables ranging from 1 to 2 vvm of aeration, 400 to 600 rpm, pH 8.0, and 30 °C, and culture medium was enriched with octanoic acid. Authors observed that the yield of the fermentation was considerably increased by stripping the product from the outlet gas by adsorption on an Amberlite XAD-4 column reaching 2 g/L/day of 2-heptanone (van der Schaft et al. 1992). This product and other methylketones are key components of various dairy flavors increasing their importance (Burdock 2010).

Indeed, lactones, methyl ketones, vanillin, benzaldehyde, alcohols, esters, terpenes, and other compounds can be produced by various fungi (mycelium or resting spores) including several fungal strains as *Fusarium* sp. and also *Aspergillus* sp., *Trichoderma*, *Ceratocystis*, *Phanerochaete*, and *Penicillium* spp., which may be cultured using solid-state or submerged fermentation (Hagedorn and Kaphammer 1994; Feron et al. 1996). Despite the diversity of compounds obtained using this technique, its production levels are very poor and thus constitute a limitation for industrial exploitation. For this reason, biotechnologists have focused on bioconversion processes that offer more economic advantages (de Carvalho and da Fonseca 2006). In this perspective, different flavor compounds have also been produced by biotransformation using fungal strain as biocatalysts, such as vanillin, important terpenes derivatives, “green notes” and mushroom flavors, fruity lactones, cheese-flavored methylketones, and others (Krings and Berger 1998; Bicas et al. 2009; Berger 2009). In this approach, some important process information is available in the recent literature, presenting the potential of *Fusarium* sp. in biotransformation processes.

Terpenes are promising substrates for biotransformation, since they are structurally related to the products obtained and can be found in nature as plant secondary metabolites and its utilization is economically viable due to the possibility of using terpene-rich by-product as substrate in flavor production process (Molina et al. 2014). The strain with the greatest potential found in the literature is recognized as *F. oxysporum* 152b, studied in a series of biotransformation processes of R-(+)- and S-(−)-limonene (Maróstica and Pastore 2007; Bicas et al. 2008, 2010a; Molina et al. 2015).

The ability of this fungal strain to bioconvert R-(+)-limonene for the production of α-terpineol was studied using cassava wastewater as culture media for the strain growth and orange essential oil as the sole carbon and energy source. Authors found that the biotransformation of R-(+)-limonene resulted in 450 mg/L of R-(+)-α-

terpineol after 3 days (Maróstica and Pastore 2007). The use of agro-industrial by-products and residues is an emergent trend for the reduction of costs inherent to bioprocess, including those related to microbial bioflavor production (Bicas et al. 2009).

Bicas et al. (2008) optimized the process conditions for the biotransformation of R-(+)-limonene to R-(+)-α-terpineol by this strain using response surface methodology. After an extensive study to evaluate process parameters, authors found a significant increase in the production of R-(+)-α-terpineol reaching up to 2.4 g/L after 72 h cultivation at 26 °C and 240 rpm. Following, Bicas et al. (2010a) described the limonene biotransformation integrated with extracellular alkaline lipase production, considering the recognized potential of *F. oxysporum* 152b for the production of this enzyme (Prazeres et al. 2006). Authors conducted a series of experiments, analyzing the product formation that reached 2 g/L and also the characteristics of the enzyme responsible for the process, observing that it would be possible to use *Fusarium* biomass stored in frozen or lyophilized conditions for α-terpineol production. Finally, the biotransformation process also occurred using the biomass resulting from the lipase production process, showing that the co-production of this enzyme and R-(+)-α-terpineol was feasible (Bicas et al. 2010a).

The last reported work that complements the series of studies employing the strain *F. oxysporum* 152B described the bioconversion process of S-(−)-limonene into limonene-1,2-diol, reaching 3.7 g/L after 72 h of process under 250 rpm and 28 °C. Comparison on cell permeabilization under anaerobic conditions and using a biphasic system was done with the recognized process of R-(+)-limonene biotransformation, identifying a limonene-1,2-epoxide hydrolase with an intracellular and cofactor-dependent nature. Interestingly, this seems to be the first report to characterize the bioconversion of R-(+)- and S-(−)-limonene using *F. oxysporum* 152b by cellular detoxification using ultrastructural analysis (Molina et al. 2015).

The biotransformation of the sesquiterpene valencene and nootkatone has also been reported for microorganisms using *F. culmorum*, *Botryosphaeria dothidea*, and *Aspergillus niger* to afford structurally interesting metabolites. After inoculation of *F. culmorum* with (+)-nootkatone as substrate for 20 days at 30 °C, it produced (11R)-11,12-dihydroxy-11,12-dihydronootkatone (47.2%) and 9β-hydroxynootkatone (14.9%), analyzed by high-resolution NMR spectral and X-ray crystallographic (Furusawa et al. 2005). The process for flavor compounds production through biotransformation of terpenes was patented by Müller et al. (2005) (WO 2005078110 A1). In this case, the freeze-dried mycelium of *F. proliferatum* was rehydrated and mixed with the substrate R-(+)-limonene for cis-(+)-carveol production. The process development is important for further application in food, cosmetic, and pharmaceutical industries. During a screening procedure, eight fungal strains were tested for their ability to convert different halolactones into new derivatives. Among the strains tested, *F. oxysporum* and *F. solani* were capable to biotransform all three substrates tested for the production of hydroxylactone after 14 days of process with good yields, 52–60% and 55–77%, respectively (Grabarczyk 2012). This product and others of the class of lactones are described as sweet, fatty, coconut, tropical, dairy odor (Burdock 2010).

In addition to these methodologies, the combination of statistical and genetic engineering tools can be considered an interesting strategy in microbial flavor production. Barros et al. (2012) performed the optimization of short-chain alkyl esters in organic solvent media using RSM based on a five-level, four-variable central composite design. Differently from the previous reports described in this section, flavor production was carried employing lyophilized *F. solani pisi cutinase* produced by a recombinant *Saccharomyces cerevisiae* SU50 strain. The products obtained were ethyl acetate, ethyl hexanoate, butyl butyrate, butyl octanoate, hexyl acetate, hexyl hexanoate, and octyl butyrate, very appreciated for their fruity aroma, being potentially used in beverages, baked goods, wines, and dairy products (Barros et al. 2012). Indeed, the production and use of enzymes from *Fusarium* sp. strains also show great potential for obtaining volatile compounds, such as estearase, lipase, and lipoxygenase (Christakopoulos et al. 1998; Stamatis et al. 1998; Dhake et al. 2013; Husson et al. 1998b). Lipoxygenase can be obtained from *F. proliferatum* and shows economic interest particularly relevant for food industry, being basis of biochemical pathway of flavor volatiles formation (Husson et al. 1998a, b). *F. oxysporum* can be considered as an interesting source for obtaining estereases, which are well suited for the production of short-chain geranyl esters by esterification or transesterification in organic solvents (Stamatis et al. 1998). Estereases were obtained from *F. oxysporum* grown on tomato skins, by-product from the tomato canning industry, as the sole carbon source under submerged and solid-state cultures. The produced enzyme catalyzes the production of geranyl acetate with yield of 68% by transesterification in organic solvents, becoming a good alternative for the synthesis and production of industrially significant acetates and esters in organic solvents (Christakopoulos et al. 1998). For example, geranyl acetate has a pleasant, flowery odor reminiscent of rose lavender and could be widely used in the food, beverage, and cosmetic industries (Burdock 2010).

Furthermore, other approaches for the production of flavor compounds using *Fusarium* sp. may include the kinetic resolution of acyclic and aromatic acetates by using the whole cells of *Fusarium proliferatum* to furnish (R)-alcohols with more than 95% of enantiomeric excess, which could be used in perfumery, cosmetic, flavor, and fragrance industries (Jadhav et al. 2016). Despite the great potential of some fungal strains for the production of bioflavors, the genus *Fusarium* sp. still has scarce information on its application in bioprocesses for the production of natural flavor compounds by de novo synthesis and biotransformation processes. In this sense, it can be considered as interesting fungal biocatalyst for further research aiming the recognition of its potential and encouraging future research in this field.

6.4.5 Pigments

In the last years, it has been suggested the use of natural pigments in industry as alternative to synthetic compounds due to increasing of the health and environmental concerns from consumer market (Mapari et al. 2010; Rodriguez-Amaya 2016;

Martins et al. 2016). Nowadays, some groups of natural pigments from plants are extensively studied due to their medical and nutraceutical potential. Curcumin from turmeric (*Curcuma longa* L.) rhizome is a natural polyphenolic yellow-orange pigment widely used in food products/supplements and showed many biological properties such as antioxidant, anti-inflammatory, and antiproliferative (Maheshwari et al. 2006; Petrova et al. 2016; Ariyarathna and Karunarathne 2016; Martins et al. 2016; Fu et al. 2014; Boonla et al. 2014; Farhangi et al. 2015). Carotenoids (α-carotene, β-carotene, β-cryptoxanthin, lycopene, lutein, and zeaxanthin) and anthocyanins (pelargonidin, cyanidin, delphnidin, peonidin, petunidin, and malvidin) are important groups of natural pigments commonly found in plants, fruits, and vegetables that can be applied in food products and exhibit many benefits to health (Rodriguez-Amaya 2016; Sancho and Pastore 2012).

However, the production and effective application of these natural pigments are limited by expensive extraction procedures and environmental factors (seasonality, humidity, and temperature). Also it is considered an extractive activity since it is necessary a large quantity of plants to obtain small pigment amounts. Other features that limit the application of natural pigments obtained from plants are associated with their chemical characteristics such as sensitivity to light, heat, or oxygen and instability in different conditions of pH (Mapari et al. 2005). An interesting alternative to overcome these problems is the use of microorganisms (bacteria, fungi, yeast, and micro-algae) in biotechnological processes for natural pigment production (Mapari et al. 2005; Dufossé et al. 2005; Tuli et al. 2015). The chemical and structural diversity of natural pigments produced by microorganisms comprises different classes with a wide range of colors. These classes include anthraquinones, hydroxyanthraquinones, naphthaquinones, carotenoids, phenolic and flavin compounds, pyrrole and azaphilone derivatives, oxopolyene, and other specific structures (Mapari et al. 2005, 2010; Duran et al. 2002; Venil et al. 2013).

Several species of *Fusarium* fungi have been described as pigment producers such as *F. solani*, *F. oxysporum*, *F. moniliforme*, *F. martii*, *F. fujikuroi*, *F. verticillioides*, *F. graminearum*, *F. culmorum*, *F. decemcellulare*, *F. bulbigenum*, *F. langsethiae*, *F. poae*, *F. sporotrichioides*, and others not identified in species level (Table 6.2) (Phelps et al. 1990; Parisot et al. 1990; Gessler et al. 2013; Pradeep et al. 2013; Duran et al. 2002; Kurobane et al. 1986; Studt et al. 2012; Boonyapranai et al. 2008; Lopes et al. 2013; Kasprowicz et al. 2013; Medentsev et al. 2005; Thrane et al. 2004). Many of *Fusarium* pigments, like bikaverin, aurofusarin, and others, are recognized as mycotoxins, and this fact is important in safety concerns, mainly considering possible applications in medical or food fields (Sasanya et al. 2008; Dvorska et al. 2001, 2002).

In terms of industrial applications, some studies describe the use of pigments produced by *Fusarium* strains in dyeing processes of diverse materials showing the potential of these compounds as alternative dyes in textile industry. Velmurugan et al. (2010) evaluated the production of different pigments by five fungal strains, including a *Fusarium* sp. strain isolated from soil, and their potential use in the dyeing of pre-tanned leather samples. This study reported that a red pigment was produced by *Fusarium* spp. when cultivated in mineral medium supplemented with

Table 6.2 Natural pigments produced by *Fusarium* strains

Producer strain	Pigment	Chemical structure	References
F. bostrycoides	Bostrycoidin (red-orange-acid conditions) (purple-alcaline conditions)		Hamilton et al. (1953)
F. javanicum	Javanicin (red)		Arnstein et al. (1946)
F. solani *F. decemcellulare*	Anhydrojavanicin (red)		Tatum et al. (1989), and Medentsev et al. (2005)
F. fujikuroi *F. oxysporum* *F. verticillioides*	Bikaverin (red)		Wiemann et al. (2009), Arndt et al. (2015), Son et al. (2008), and Lazzaro et al. (2012)
F. culmorum *F. graminearum* *F. culmorum* *F. langsethiae* *F. poae* *F. sporotrichioides*	Aurofusarin (yellow-acid conditions) (red/purple-alcaline conditions)		Ashley et al. (1937), Frandsen et al. (2006), and Thrane et al. (2004)

(continued)

Table 6.2 (continued)

Producer strain	Pigment	Chemical structure	References
F. oxysporum	2-acetyl-3,8-dihydroxy-6-methoxy anthraquinone (red)		Nagia and EL-Mohamedy (2007)
	3-acetyl-2,8-dihydroxy-6-methoxy anthraquinone (red)		
Fusarium sp. ZZF60	6,8-dimethoxy-1-methyl-2-(3-oxobutyl) anthrakunthone (yellow)		Huang et al. (2010)
F. oxysporum *F. solani*	5-*O*-methyljavanicin (red)		Tatum et al. (1985) and Kimura et al. (1981)
F. moniliforme KUMBF1201	2-(4-((3,5)-14-aminotetradeca-3,5-dienyloxy)butyl)-1,2,3,4-tetrahydroisoquinolin-4-ol (pink)		Pradeep et al. (2015)

glucose (30 g/L). Previously, Nagia and El-Mohamedy (2007) showed the feasibility for dyeing of wool using two natural anthraquinone compounds with red color from *F. oxysporum* strain, isolated from roots of citrus. The bioprocess for pigment production by this fungus was carried out using mineral medium with 20 g/L of glucose as a carbon source (Tatum et al. 1985). In addition, another *F. oxysporum* isolated was able to produce six naphthoquinone pigments when cultivated in similar conditions. These pigments exhibited different shades of red after the purification process, and the compound with highest concentration was a red-brown naphthoquinone (Tatum et al. 1987).

Two processes for obtaining blue pigments from *F. oxysporum* CCT7620 and their use in dyeing of fabric and plastic material were patented recently (BR102013015305 and BR102013027036) and mentioned by Bicas and Silva (2013a, b). In this process, the blue pigment was produced in solid-state fermentation or submerged fermentation with rice cooked as substrate for growth and concomitant of the pigment. The biotechnological production of pigments and other secondary metabolites is influenced by diverse factors such as substrate type, pH, oxygen and carbon dioxide levels, medium composition, nitrogen and carbon sources, agitation, temperature, and the development stage of the fungi used (Sagaram et al. 2006; Akilandeswari and Pradeep 2016; Souza et al. 2016). However, these factors can present different effects for each *Fusarium* species and consequently in their secondary metabolite profile (Sørensen and Sondergaard 2014). The effect of several production medium, temperature, pH, incubation period, carbon and nitrogen sources, amino acids, and metal salts on pigment and biomass production by *F. moniliforme* strains (KUMBF1201, KUMBF1202, KUMBF1206, and KUMBF1207) in submerged fermentation was evaluated in study performed by Pradeep and Pradeep (2013). Among the six liquid media tested, maximum biomass and pigment production were achieved using commercial potato dextrose broth (PDB). In addition, the best conditions to optimum pigment biosynthesis were with basal medium, enriched with 20 g/L of glucose as carbon source and 10 g/L of peptone as nitrogen source, at 28 °C and pH 5.5, in an incubation period of 8 days.

The influence of pH, nitrogen, and phosphorus limitation on pigment and biomass production by *F. bulbigenum*, *F. graminearum*, and *F. decemcellulare* in submerged fermentation was evaluated. Medentsev et al. (2005) proved that different pigments can be obtained depending of the conditions in biotechnological process. In acid conditions, *F. decemcellulare* produced soluble extracellular pigments (naphthoquinones), whereas *F. bulbigenum* biosynthesized bikaverin. When in alkaline conditions, *F. graminearum* and *F. decemcellulare* were able to produce aurofusarin. Furthermore, the inhibition of fungal growth for all strains under nitrogen and phosphorus limitation was observed, although aurofusarin was still produced by both fungal strains. The authors suggested that the biosynthesis of pigments is initiated during the growth inhibition and transition to the stationary phase of fungi during submerged fermentation conditions (Medentsev et al. 2005).

Lale and Gadre (2016) investigated the influence of different carbon and nitrogen sources on bikaverin production by a mutant strain of *F. fujikuroi* NCIM 1019 in shake flask cultures. The bioprocess was carried out at 28 °C and 200 rpm for 5 days

using basal fermentation medium. The carbon sources tested were cellobiose, fructose, galactose, glucose, lactose, maltose, sucrose, xylose, and soluble starch, whereas the organic nitrogen sources used were meat peptone, soy peptone, yeast extract, defatted cottonseed meal, and soy bean meal. Interesting results of bikaverin production were achieved in media containing glucose (around 3.0 g/L) or defatted cotton seed meal (about 4 g/L). The combination of these nutrient sources resulted in 6.3 g/L of bikaverin. Using agro-industrial by-products as source of nutrients in submerged fermentation process, *F. graminearum* IFL3 was able to produce yellow pigments, observed by the maximum absorption in wavelengths around 400 nm. In this study, all residues were used at 10 g/L and cultivated at 30 °C, 125 rpm for 7 days. After chemical characterization of metabolites presents in organic extracts, the presence of diacetoxyscirpenol, fusarenone X, 15-acetoxyscirpenol, and neosolaniol were observed in addition to four mycotoxins commonly described as produced by *Fusarium* strains (Lopes et al. 2013).

Beyond the color characteristics of the pigments produced by fungi of *Fusarium* genus, some of these compounds may present biological properties. In a recent study performed Pradeep et al. (2015), it was demonstrated the larvicidal activity of isoquinoline pigment produced *F. moniliforme* KUMBF1201 against third- and fourth-instar larvae of *Aedes aegypti* and *Anopheles stephensi*. Previously, Prakash et al. (2010) suggested that the larvicidal effects of *F. oxysporum* against *Anopheles stephensi* and *Culex quinquefasciatus* in laboratory assay would be associated to the secondary metabolites and toxins produced by this strain.

Recently, different organic extracts ((ethyl acetate (EtOAc) and n-butanol (n-BuOH)) from *F. oxysporum* culture filtrate were evaluated against plant parasitic nematodes *Meloidogyne incognita* and *Rotylenchulus reniformis*. The concentrate extracts showed high antinemic activity for *M. incognita*, with LC50 values of the 56.2 and 97.49 μg/mL to EtOAc and n-BuOH extract, respectively. When these extracts were tested against *R. reniformis*, moderate activity was observed with LC50 values of 134.5 (EtOAc) and 189.2 9 μg/mL (BuOH). As the best results were obtained for EtOAc extract, its purification and chemical characterization were performed. In this extract, five different pigments were identified as bikaverin, fusarubin, and their derivatives 3-O-methyl-8-O-methylfusarubin, 8-O-methyl fusarubin, and anhydrofusarubin. Moreover, the individual antinemic activity was evaluated for these compounds, and fusarubin exhibited the highest potential for the nematodes tested with LC50 values of 248.9 μg/mL to *M. incognita* and 301.6 μg/mL to *R. reniformis*. Therefore, the pigments produced by *Fusarium* strains can be considered potential biocontrol agents against vector mosquitoes or nematodes of diverse diseases that can affect human or plants (Kundu et al. 2016).

Fusarium pigments also can exhibit antimicrobial activity against several important pathogenic microorganisms, including *Staphylococcus aureus*, *Streptococcus pyogenes*, *Escherichia coli*, *Klebsiella pneumoniae*, *Salmonella typhi*, *Proteus vulgaris*, *Serratia marcescens*, *Pseudomonas aeruginosa*, and *Candida albicans* (Baker et al. 1990; Frandsen et al. 2016). Deshmukh et al. (2014) verified the antibacterial activity of organic extracts obtained from broth of *Fusarium* species after cultivation in minimal medium with 10% glucose at 30 °C, 120 rpm for 7 days.

This extract exhibited antibacterial spectrum against pathogenic and multidrug resistant bacteria strains. After purification and chemical characterization, the antimicrobial compound was identified as bikaverin.

Some studies have described the potential use of natural pigments from *Fusarium* sp. for therapeutic applications. The yellow pigment anthraquinone produced by *Fusarium* sp. ZZF60, isolated from mangrove, exhibited cytotoxicity activity against Hep2 and HepG2 cells (Huang et al. 2010). Nirmaladevi et al. (2014) described the protective effects of bikaverin produced by *F. oxysporum*, isolated from rhizosphere soil of tomato plant, on oxidative stress using human neuroblastoma SH-SY5Y cells and showed that this pigment acts through the anti-apoptotic mechanism to attenuate H2O2-induced neurotoxicity. Thus, due to chemical and biological properties of natural pigments from *Fusarium* sp., these compounds may be applied not only as food additive but also in industrial and medical fields. Nevertheless, more studies are required in order to evaluate biosafety and to optimize the biotechnological process aiming the increase in product concentration.

Bioactive secondary metabolites. Fungal organisms synthesize a broad range of unique bioactive compounds with low molecular weight, of which can be highlighted a remarkable variety of metabolites with pharmacological effects, including the β-lactams (penicillin and cephalosporin), several statins (lovastatin, mevinolin, compactin, pravastatin, atrovastin), and immunosuppressant (cyclosporin and ergotamine) (Bérdy 2005; Misiek and Hoffmeister 2007; Fox and Howlett 2008; Du and Lou 2009). In general, these compounds are classified as polyketides, non-ribosomal peptides (NRPs), terpenes, or alkaloids, which clustered gene expression involves a multidomain responsible for encoding multimodular enzymes named polyketide synthases (PKSs), non-ribosomal peptide synthetases (NRPSs), or a hybrid PKS-NRPS enzyme as well as the prenyltransferases and terpene cyclases (Keller et al. 2005; Brakhage and Schroeckh 2011; Brakhage 2013). Both PKs and NRPS are biosynthesized through acyl coenzyme A molecules (mainly malonyl CoA and acetyl CoA) and amino acids, while the PKS-NRPS hybrids consist of a fungal iterative type I PKS fused to a single NRPS module that is sometimes truncated (Brakhage 2013).

As well-known, the evolution of these so-called secondary metabolites occurred because microorganisms used them as chemical signals for communication, to resist in unfavorable survivable environments or to inhibit the growth of competitors (Zhong and Xiao 2009; Brakhage 2013). Some metabolites are exploited due to their biological activities (agricultural, pharmacological, or medical activity), while others are involved in diseases (toxic functions, e.g., mycotoxin and aflatoxins), resulting from fungi interactions with plants or animals. In this context, terrestrial, marine, or endophytic species have been isolated as sources of new bioactive compounds including chemopreventive agents possessing the bioactivity of immunomodulatory, anticancer, and others (Bérdy 2005; Keller et al. 2005).

Members of *Aspergillus*, *Penicillium*, *Fusarium*, *Trichoderma*, *Phoma*, *Alternaria*, and *Acremonium* genera have been described as producers of polyketides, terpenoid metabolites, steroids, indole alkaloids, and peptides whose the notable representatives of drugs developed are cytochalasin B (phomin) from *Phoma exigua*,

brefeldin A from *P. brefeldianum*, verrucarin A from *Myrothecium verrucaria*, anguidine (diacetoxyscirpenol) from *F. diversisporium*, and famous immunosuppressive drug cyclosporine A from *Tolypocladium inflatum* (Hanson 2008; Zhong and Xiao 2009). Particularly, the *Fusarium* species are able to synthesize a variety of structures which may have a positive and negative impact on the health aspects. Trichothecenes, zearalenone, and fumonisins are the most known metabolites that have been recognized as harmful, but they may produce other compounds such as pigments, antibiotics, and phytotoxins with positive aspects (Nelson et al. 1993; Waśkiewicz and Stępień 2012).

Regarding biological effects, some of them show antibacterial (nigrospoxydon A and nigroporapyrone A), antifungal (fugerin), and cytotoxic activities (neomangicols) as well as anti-*Helicobacter pylori* (methylsulochrin) activities (Trisuwan et al. 2010). Table 6.3 exemplifies some of the main compounds with biological potential obtained from the *Fusarium* genus. Recently, Shiono et al. (2016) reported three novel benzenediol lactone derivatives by *F. solani* T-13 isolated from a dead branch. The compounds showed weak cytotoxicity against promyelocytic leukemia HL60 cells (IC50 >10 mM) and were able to effect Ca2+ signal transduction, proved by the growth-restoring activity in a mutant strain of *Saccharomyces cerevisiae* that could not grow in the presence of high concentration of CaCl2. Yang et al. (2011, 2012a, b) reported for the first time the production of fusaroside, a unique glycolipid from the genus, and the new azaphilone and isocoumarin derivatives, named fusarone and *fusariumin* from *Fusarium* sp. All of these compounds were obtained from endophytic species of *Fusarium* isolated from leaves of *Melia azedarach* L. and displayed significant growth inhibitory activity against the brine shrimp (*Artemia salina*).

Regarding the anticancer potential, some studies have attributed the antiproliferative and cytotoxic activity of *F. incarnatum* and *F. solani* due to the presence of alkaloids, pyrones, and quinones (Ding et al. 2012; Trisuwan et al. 2013; Takemoto et al. 2014). In the same way, Ibrahim et al. (2016a) demonstrated the antimicrobial and cytotoxic activity of fusarithioamide A, produced by the endophytic fungus *F. chlamydosporium*, isolated from the leaves of *Anvillea garcini* (Burm.f.) DC. (Asteraceae). A potent activity against *Bacillus cereus*, *Staphylococcus aureus*, *Escherichia coli*, and *Candida albicans* with MIC values of 3.1, 4.4, 6.9, and 2.6 μg/mL, respectively, was reported. Regarding inhibition of cancer cell line growth, fusarithioamide A showed cytotoxic activity against SK-MEL, KB, BT-549, and SKOV-3 with IC50 0.4–1.90 μM compared to doxorubicin (IC50 = 0.046 and 0.313 μM).

Five new tetracyclic triterpenoids, named integracides F–J, from *Fusarium* sp. obtained from the roots of *Mentha longifolia* L. (Labiatae) were also isolated. These compounds showed antiproliferative effect (BT-549-1.82. SKOV-3 and KB cell lines; IC50 = 1.82–1.97 μM, 0.16–1.32 μM, and 0.18 μM, respectively) and significant antileishmanial activity (IC50 = 2.53–4.75 μM) (Ibrahim et al. 2016b, c). High yield (19.04 ± 0.82 g/kg dw) of sambacide, another tetracyclic triterpenoid, was obtained after incubation of *F. sambucinum* for 20 days in solid-state fermentation. This compound exhibited antibacterial activity against *S. aureus* and *E. coli* with

Table 6.3 Bioactive secondary metabolites produced by *Fusarium* sp.

Producer strain	Secondary metabolites	Chemical structure	Biological properties	References
F. solani	Fusapyrone A and B (Pyrones)		Cytotoxic activity	Trisuwan et al. (2013)
	Dihydronaphthoquinone derivatives (Quinones)			Takemoto et al. (2014)
	Camptothecin (Pentacyclic quinoline alkaloid)			Venugopalan and Srivastava (2015), Venugopalan et al. (2016)

(continued)

Table 6.3 (continued)

Producer strain	Secondary metabolites	Chemical structure	Biological properties	References
F. chlamydosporium	Fusarithioamide A (Benzamide derivative)		Anti-leishmanial and cytotoxic activities	Ibrahim et al. (2016a)
F. tricinctum	Enniatin (Cyclic depsipeptide)		Antibacterial and antifungal activities and cellular proliferation inhibitor	Zaher et al. (2015)
			Antibacterial and ant-leshmanial activities	

MIC values of 16 and 16 μg/mL, respectively (Dong et al. 2016). Due to the great potential as an antimicrobial agent, the bioprocess production was patented (CN106117293) (Ding et al. 2016). Although fusarielins isolated from *Fusarium* sp. remains an underexploited group of secondary metabolites, some studies have been correlating this mycoestrogen as protection agent against human breast cancer cell lines (Sondergaard et al. 2012). Certainly, further research with *Fusarium* secondary metabolites should be conducted in order to study their use in biotechnology, genetic engineering, metabolic technology, and microbial fermentation process leading to the design of new drugs and further clinical trials. In addition, it is necessary to gain deeper knowledge about the mechanism of action as well as the metabolic pathways enrolled in biological effects through in vitro and in vivo studies.

6.5 Conclusion and Future Prospects

Fusarium species have been reported in many biotechnological processes for natural compound production. The ability of these fungi to produce a wide variety of enzymes and secondary metabolites makes possible their use in the most diverse fields, such as food, cosmetic, cleaning agents, biofuels, and pharmacy. The major challenges involving the use of *Fusarium* sp. strains are the low yields of products obtained and also the proved pathogenicity and mycotoxin production. In this context, industrial biotechnology and genetic engineering are promising strategies to overcome these concerns. By heterologous expression of *Fusarium* sp. genes in yeasts and bacteria, the process feasibility is increased, since these microorganisms are of easier manipulation and some of them recognized as safe (GRAS). Moreover, the combination of genetic engineering and application of statistical tools can lead to great increases in yield and productivity. It can be highlighted that in most of the process using *Fusarium* sp., it is possible to use agro-industrial wastes and by-products for biomass growth and/or as alternative substrate for the generation of value-added compounds, resulting in a more sustainable process, reducing overall process costs, and increasing the upscale potential. Thus, the potential of *Fusarium* strains and its derivatives for industrial application needs more concret studies and real application.

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Chapter 7
Industrially Important Enzymes from Fungal Endophytes

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Abstract The relationship between plant endophyte is noted for mutualism and balanced antagonism between endophytic virulence and plant defensive response. The host plant produces many toxic substances to limit the growth of endophytes, but during the long period of coevolution, endophytes also have gradually formed several tolerant mechanisms toward host metabolites by producing exoenzymes and mycotoxins. These enzymes include pectinase, cellulase, lipoidase, proteinase, phenol oxidase, and lignin catabolic enzymes. When host plants die, the fungi utilize the carbon source plant residues such as glucose, oligosaccharide, cellulose, hemicellulose, lignin, keratin, pectin, lipid, and protein and decompose effectively. These enzymes may also degrade macromolecule compounds into small molecules or convert more toxic substances into less toxic in order to increase their adaptability. The large amounts of residual plant biomass (lignocellulosic) which are considered as waste can potentially be converted with the mediation of microbes into various different value-added products including biofuels, chemicals, cheap energy sources for fermentation, improved animal feeds, and human nutrients. Lignocellulolytic enzymes also have significant potential applications in various industries such as chemical, fuel, food, brewery and wine, animal feed, textile and laundry, pulp and paper, and agriculture. In this review, we have reported the ability of endophytic fungi in the production of different enzymes of immense values in agriculture, medicine, and other industries.

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7.1 Introduction

Fungi being ubiquitous in distribution are highly successful in survival because of their great plasticity and physiological versatility to secrete a wide range of enzymes involved in the breakdown of complex polymers which enable them to use many biomass constituents as energy and carbon sources. Fungi thrive well in unfavorable habitats with environmental extremes because of their efficient enzyme systems. Production of extracellular enzymes is one of the varied mechanisms of fungi in adaptability, survival, and utilization of their ecological niche conditions (Gopinath et al. 2005). The ability to secrete extracellular protein makes filamentous fungi attractive hosts for the production of proteins having immense value in agriculture, paper, pulp, and pharmaceutical industries (Carlsen and Nielsen 2001). Lignocellulose is the major structural component of plants and represents a major source of renewable organic matter (Howard et al. 2003). Wood is a complex of many types of chemicals whose concentrations vary among and within the species. The chemical constituents of wood can be grouped into primary metabolites such as soluble sugars, lipids, and peptides and the major storage compound starch; cell wall components such as hemicellulose, cellulose, and lignin; and minerals and exclusive of extractable primary metabolites. Studies of physiological variation among the fungi that degrade wood cotton and various food products as well as soil-inhabiting microorganisms, it often is desirable to determine and compare the cellulolytic activity of various species. Conventional methods to determine such activity involve either assessment of activity in culture filtrate or direct study of growing organisms (Rautela and Cowling 1966).

In the meantime, huge amounts of lignocellulosic wastes are generated through several anthropogenic activities like forestry and agricultural practices, paper-pulp industries, timber industries, and many agro industries, and they pose an environmental pollution problem (Howard et al. 2003). The lignocellulosic material of plants consists of three main components, namely, cellulose, hemicellulose, and lignin. Cellulose is a linear homopolymer of glucose units linked with β-1,4-glycosidic bonds. Naturally, cellulose is catalyzed by extracellular enzymes cellobiohydrolases, endoglucanases, and glucosidases produced by fungi and bacteria. Hemicelluloses are heteropolysaccharides consisting of short branched chains of hexoses, i.e., mannose units in mannans and pentose units in xylans. These hemicelluloses are degraded by the enzymes endoxylanases and endomannanases. The main extracellular enzymes are heme-containing lignin peroxidase, manganese peroxidase, and Cu-containing laccase.

A diverse spectrum of lignocellulolytic microorganisms mainly fungi (Falcon et al. 1995; Baldrian and Gabriel 2003) and bacteria (Vicuna 1988) have been isolated and identified. *Trichoderma reesei* and its mutants are studied extensively and are widely employed for the commercial production of hemicellulases and cellulases (Nieves et al. 1998). The white rot fungi belonging to the basidiomycetes are

the most efficient and extensive lignin degraders. *Phanerochaete chrysosporium* was reported as the best-studied lignin-degrading fungus, producing copious amounts of a unique set of lignocellulolytic enzymes (Howard et al. 2003). Fungi can produce both intracellular and extracellular enzymes. All fungi are heterotrophic and rely on carbon compounds synthesized by other living organisms. Small molecules like mono- or disaccharides, fatty acids, and amino acids can easily pass through the cell membrane, but larger molecules like cellulose, hemicellulose, lignin, statin, and pectin cannot pass through it. Fungi secrete extracellular enzymes. Production of extracellular enzymes is faster and easier to be extracted when compared to intracellular enzymes (Hankin and Anagnostakis 1975). The large amounts of residual plant biomass (lignocellulosic) which are considered as waste can potentially be converted with the mediation of microbes into various different value-added products including biofuels, chemicals, cheap energy sources for fermentation, improved animal feeds, and human nutrients. Lignocellulolytic enzymes also have significant potential applications in various industries such as chemical, fuel, food, brewery and wine, animal feed, textile and laundry, pulp and paper, and agriculture (Sahay et al. 2017; Shukla et al. 2016; Yadav et al. 2016b; Levine 1996; Howard et al. 2003).

Endophytic fungi have been considered as repository of novel bioactive substances. The substances produced by endophytic fungi belong to terpenoids, steroids, xanthones, quinines, phenols, isocoumarins, benzopyrones, tetralones, cytochalasins, and other derivatives (Schulz et al. 2002; Suman et al. 2016; Yadav et al. 2018) (Table 7.1). Endophytic fungi represent a reservoir for discovering new compounds such as antibiotics, antioxidants, immunomodulators, and anticancer and antiparasitic compounds having importance in the pharmaceutical and agrochemical industries (Corrêa et al. 2014). Several reviews have emphasized the need to routinely include endophytic fungi in the screening of organisms for bioactive metabolites and novel drugs (Tan and Zou 2001; Borges et al. 2009; Suryanarayanan et al. 2012). Most studies so far have thus focused on the production or at least of the perspectives of production of such compounds by endophytic fungi (Marlida et al. 2000a, b; Chen et al. 2011). Endophytic fungi produce enzymes (hydrolytic and oxidative) such as amylases, lipases, and proteases as part of their mechanism to overcome the defense of the host against microbial invasion and to obtain nutrients for their development (Verma et al. 2016b; Torres et al. 2003; Sunitha et al. 2012; Rana et al. 2016a, b, 2017). Many of these enzymes are involved in the degradation of components of lignocellulosic materials. Endophytic fungi produce hydrolytic enzymes like xylanases and cellulases to degrade the lignocellulosic fibers and the oxidative ligninolytic enzymes such as laccases, ligninases, and peroxidases to degrade lignin. The ability of endophytic fungi to degrade the complex structure of lignocelluloses makes them potentially useful in the exploration of the lignocellulosic biomass for the production of fuel, ethanol, and industrially useful enzymes such as lipases, phytases, amylases, and proteases (Shukla et al. 2016; Verma et al. 2012; Yadav et al. 2012, 2014, 2016a).

Table 7.1 Enzymes produced by endophytic fungi from different host plants

Endophytic fungi	Host plant	Enzyme	Reference
Acremonium zeae, *Acremonium* sp.	Corn	Cellulases and hemicellulases	Almeida et al. (2011)
Cladosporium cladosporioides, *Nigrospora sphaerica*, *Colletotrichum gloeosporioides*	*Costus igneus*, *Lawsonia inermis*	Amylase, cellulose, protease	Amrita et al. (2012)
Curvularia brachyspora	*Adhatoda vasica*	Amylase, laccase, lipase	Amrita et al. (2012)
Curvularia vermiformis, *Xylaria* sp.	*Coleus aromaticus*	Cellulose, lipase, protease Amylase, laccase, protease	Amrita et al. (2012)
Drechslera hawaiiensis	*Adhatoda vasica*	Amylase, lipase, protease	Amrita et al. (2012)
Colletotrichum crassipes, *Colletotrichum falcatum*	*Lawsonia inermis*	Amylase, protease Lipase, protease	Amrita et al. (2012)
Phyllosticta sp.	*Adhatoda vasica*, *Lawsonia inermis*	Amylase, lipase	Amrita et al. (2012)
Acremonium terricola, *Cladosporium cladosporioides*, *Fusarium lateritium*, *Nigrospora sphaerica*, *Penicillium aurantiogriseum*, *Pestalotiopsis guepinii*, *Xylaria* sp.	*Opuntia ficus–indica* Mill.	Cellulose, protease, xylanase	Amrita et al. (2012)
Aspergillus fumigates, *A. niger*		Cellulose, xylanase	Baffi et al. (2012)
Aspergillus japonicus	*Opuntia ficus–indica* Mill.	Cellulose, pectinase, protease, xylanase	Bezerra et al. (2012)
Monodictys castaneae	*Opuntia ficus–indica* Mill.	Xylanase	Bezerra et al. (2012)
Phoma tropica, *Phomopsis archeri*, *Tetraploa aristata*, *Xylaria* sp.	*Opuntia ficus–indica* Mill.	Protease, xylanase	Bezerra et al. (2012)
Talaromyces flavus	*Potentilla fulgens*	Lipase, protease, xylanase	Bhagobaty and Joshi (2012)
Mortierella hyalina	*Osbeckia stellata*	Cellulase, lipase, protease, xylanase	Bhagobaty and Joshi (2012)
Paecilomyces variabilis	*Osbeckia chinensis*	Amylase, lipase, protease, xylanase	Bhagobaty and Joshi (2012)
Penicillium sp.	*Camellia caduca*	Cellulase, lipase, protease, xylanase	Bhagobaty and Joshi (2012)
Penicillium sp.	*Schima khasiana*	Cellulase, lipase, protease, xylanase	Bhagobaty and Joshi (2012)

(continued)

Table 7.1 (continued)

Endophytic fungi	Host plant	Enzyme	Reference
Acremonium zeae	*Zea mays*	Xylanase	Bischoff et al. (2009)
Colletotrichum musae	*Musa cavendish*	Acid phosphatase	Maccheroni and Azevedo (1998)
Penicillium sp.	*Centella asiatica*	Cellulase	Devi et al. (2012)
Penicillium sp.	*Centella asiatica*	Cellulase	Devi et al. (2012)
Trichoderma sp., *Penicillium* sp., *Aspergillus* sp.	*Latrunculia corticata*	Cellulases	El-Bondkly and El- Gendy (2012)
Penicillium spp.	*Dendronephthya hemprichii*	Keratinase	El-Gendy (2010)
Chaetomium globosum	*Glinus lotoides*	Laccase	El-zayat (2008)
Colletotrichum sp.	*Abelmoschus esculentus*	B-galactosidase, rhamnogalacturonan lyase, acetyl esterase	Grünig et al. (2008)
Melanconium apiocarpum		Laccase, amylase, cellulase	Guo et al. (2008)
Periconia sp.		B-glucosidase	Harnpicharnchai et al. (2009)
Discosia sp.	*Calophyllum inophyllum*	Amylase	Hegde et al. (2011)
Piriformospora indica		Amylase	Kumar et al. (2012)
Epichloe sp.	*Poa ampla*	β-1,6-Glucanase	Li et al. (2004)
Fusarium oxysporum, *Gibberella* sp.	*Ophiopogon japonicus*	Peptide deformylase	Liang et al. (2012)
Periconia atropurpurea		Esterase	Lisboa et al. (2013)
Mycelia sterilia	*Rhus chinensis* Mill.	Laccase	Lumyong et al. (2002)
Epichloe sp.	*Poa ampla*	β-1,6-Glucanase	Maccheroni and Azevedo (1998)
Acremonium sp. *Fusarium* sp.	*Acrostichum aureum*	Amylase, cellulase, lipase	Maria et al. (2005)
Alternaria chlamydospora, *Pestalotiopsis* sp.	*Acanthus ilicifolius*	Cellulase, lipase, protease Amylase, cellulase, lipase, protease	Maria et al. (2005)
Alternaria sp., *Aspergillus* sp.	*Acrostichum aureum*	Cellulase, lipase, protease	Maria et al. (2005)
Acremonium sp.	Forest trees in Malaysia	Glucoamylase	Marlida et al. (2000a, b)
Fusarium verticillioides, *Rhizoctonia* sp.	*Glycine max*	Phytase	Marlida et al. (2010)
Epichloe sp.	*Poa ampla*	B-1,6-Glucanase	Moy et al. (2002)

(continued)

Table 7.1 (continued)

Endophytic fungi	Host plant	Enzyme	Reference
Colletotrichum sp.	*Cinnamomum iners* *Camellia sinensis*	Cellulase, mannose, protease, xylanase	Moy et al. (2002)
Pestalotiopsis sp.	*Manglietia garrettii*	Cellulose, mannase	Moy et al. (2002)
Phoma sp.	*Garcinia cowa*	Cellulose, mannase, protease	Moy et al. (2002)
Xylaria sp.	*Trichilia connaroides*	Cellulose, mannase, xylanase, protease	Moy et al. (2002)
Phomopsis sp.	*Garcinia cowa* *Trichilia connaroides* *Cinnamomum iners*	Cellulose, mannase, xylanase	Moy et al. (2002)
Fusarium sp., *Cercospora* sp.	*Baccharis dracunculifolia*	Phenoloxidases	Onofre and Steilmann (2012)
Phoma herbarum, *Schizophyllum commune*	*Piper hispidum* SW.	Protease	Orlandelli et al. (2015)
Bjerkandera sp.	*Drimys winteri*	Cellulose, phenoloxidase	Oses et al. (2006)
Acephala applanata	Conifer roots	Amylases, laccases, proteases	Reddy et al. (1996)
Aspergillus niger, *Trichoderma atroviride*, *Alternaria* sp., *Annulohypoxylon stygium*, *Talaromyces wartmanni*	*Eucalyptus benthamii* *Platanus orientalis* *Glycine max* *Solanum tuberosum* *Saccharum officinarum*	Xylanase, hemicellulases	Robl et al. (2013)
Acremonium typhinum	*Poa ampla*	Proteinase	Sieber et al. (1991)
Cylindrocephalum sp.	*Alpinia calcarata* (HAW.) Roscoe	Amylase	Sunitha et al. (2012)
Cylindrocephalum sp.	*Alpinia calcarata*	Amylase	Sunitha et al. (2012)
Trichoderma harzianum	*Sargassum wightii*	Xylanase	Thirunavukkarasu et al. (2015)
Lasiodiplodia theobromae	Coconut	Lipase	Venkatesagowda et al. (2012)
Monotospora sp.	*Cynodon dactylon*	Laccase	Wang et al. (2006)
Neotyphodium lolii	*Poa ampla*	β-1,6-Glucanase	Wang et al. (2006)
Epichloe festucae	*Poa ampla*	β-1,6-Glucanase	Wang et al. (2006)
Monotospora sp.	*Cynodon dactylon*	Laccase	Weihua and Hongzhang (2008)

7.2 Industrially Important Enzymes

7.2.1 Xylanases

Xylanases randomly hydrolyzed the B-1,4-glycosidic bonds of xylan, the major plant cell wall polysaccharide component of hemicelluloses. Xylan has a complex structure consisting of B-1,4-linked xylose residues in the backbone to which short side chains of O-acetyl, α-Larabino furanosyl, D-α-glucuronic, and phenolic acid residues are attached (Coughlan and Hazlewood 1993). A variety of microorganisms comprising mainly fungi and bacteria are reported to produce xylanases that can degrade β-1,4-xylan in a random fashion yielding a series of linear and branched oligosaccharide fragments (Sunna and Antranikian 1997). Xylanases find specific application in jute fiber upgradation (Knob et al. 2010). Endophytic fungi were reported to be a good producer of xylanases when grown on xylan as the substrate (Medeiros et al. 2000). Xylanases have potential applications in various industrial processes such as improvement of digestibility of animal feedstock and clarification of juices and facilitating the release of lignin from pulp and reducing the amount of chlorine required for bleaching in paper and pulp industry (Beg et al. 2001; Wong and Saddler 1993; Saxena et al. 2015; Yadav et al. 2014, 2017).

7.2.2 Cellulases

Cellulose is one of the most abundant renewable polymers composed of p-1, 4 linked glucose molecules. Cellulolytic microorganisms produce a complex array of glycosyl hydrolases during the growth of cellulosic substrates. Endoglucanases and cellobiohydrolases also called cellulases are responsible for hydrolysis of cellulose. Hydrolysis of hemicellulose, a mixed polymer, occurs via the action of xylanases, mannanases, and other hydrolytic enzymes with broad substrate specificity. Cellulases possess complex enzyme system comprising endo-1, 4-β-D-glucanase, exo-1,4-β-glucanase, and exo-1,4-D-glucosidase. These enzymes together with hemicellulases and pectinases are employed in the processing of lignocellulosic materials (Nigam and Singh 1995). Cellulases and xylanases are however found applications in several other areas like in the textile industry for fiber treatment process (Suman et al. 2015; Pandey et al. 1999). Cellulases are synthesized by many cellulolytic filamentous fungi such as the *Chaetomium*, *Fusarium*, *Myrothecium*, *Trichoderma*, *Penicillium*, and *Aspergillus* species (Yadav et al. 2018; Justin 1989; Verma et al. 2012). Cellulose biodegradation by cellulases and cellulosomes produced by numerous microorganisms evoked interest among the researchers to find an eco-friendly tool in several agricultural and waste treatment processes and was widely used to produce sustainable bio-based products and bioenergy to replace

depleting fossil fuels (Melillo et al. 1989). Filamentous fungi typically *Trichoderma* and *Aspergillus* species are the well-known and efficient producers of plant cell wall-degrading systems consisting of three classes of enzymes: endoglucanases, cellobiohydrolases, and 3-glucosidases. Members of all these three classes are necessary for the degradation of cellulose (Bhat 2000). In a study, endophytes such as *Aspergillus niger*, *Trichoderma atroviride*, *Alternaria* sp., *Annulohypoxylon stygium*, and *Talaromyces wortmannii* produced the hemicellulases and other related enzymes suitable for lignocellulosic biomass degradation (Robl et al. 2013). Similarly, in a recent study, endophytic *Acremonium* strictum isolated from Brazilian biome produced the cellulase on different substrates (Goldbeck et al. 2013). Endophytic species belonged to *Colletotrichum* and *Alternaria* were described as cellulose producers with the additional capability of producing substantial amounts of lipids when cultured on rice straw and wheat bran in solid state fermentation (Dey et al. 2011). Bischoff et al. (2009) reported the ability of production of hemicellulase from endophytic *Acremonium zeae* and its capacity to hydrolyze corn arabinoxylan. Two species of *Acremonium* were able to produce cellulases and hemicellulases in submerged culture (SC) and in solid-state fermentation (SSF), using different carbon sources (Almeida et al. 2011).

7.2.3 Laccases

Laccases are glycosylated polyphenol oxidases (Thurston 1994). These enzymes find important commercial applications in the pulp and paper industry, animal biotechnology biotransformation, and detoxification of phenolic pollutants (Brenna and Bianchi 1994; Breen and Singleton 1999). Laccase production is a common feature of many basidiomycete fungi particularly those associated and involved in wood decay or terminal stages of decomposition (Gianfreda et al. 1999). The genus *Trametes* seems to be one of the most efficient laccase producers (Jang et al. 2002). Endophytes are a rich and reliable source of bioactive metabolites with huge medical, agricultural, and industrial potentials (Tan and Zou 2001). Although enzymes vary from isolate to isolate, the endophytic fungi known to produce enzymes, such as pectinases, xylanases, cellulases and lipases, proteinases, and phenoloxidases, are necessary for penetrating and colonizing their plant hosts (Tan and Zou 2001; Schulz et al. 2002).

Wang et al. (2006) reported the laccase production by endophytic *Monotospora* sp. isolated from *Cynodon dactylon*. In this study, maltose (2 g/L) and ammonium tartrate (10 g/L) were found to be the most suitable carbon and nitrogen sources, respectively, for enzyme production. Chen et al. (2011) reported the laccase production from endophytic *Pestalotiopsis* sp. isolated from sea mud collected from East China Sea under submerged and solid-state fermentation using various lignocellulosic by-products as substrates. The endophytic fungus *Phomopsis liquidambari* that grows on phenolic 4-hydroxybenzoic acid as the sole carbon and energy source is able to produce the ligninolytic enzymes laccase and lignin peroxidase when cultured in submerged fermentation (Chen et al. 2013).

7.2.4 Proteases

Proteases (serine protease, cysteine protease, aspartic protease, and metalloprotease) are the most important class of enzymes that catalyze the total hydrolysis of proteins and have been studied extensively since the advent of enzymology (Nielsel and Oxenboll 1998). The inability of the plant and animal protease to meet current world demands has led to an increased interest in microbial proteases. Proteolytic enzymes find application in a number of biotechnological processes, viz., in food processing and pharmaceuticals, leather industry, and detergent industry (Joo et al. 2003) (Table 7.1). Fungi are known to produce a wider variety of enzymes over a wide pH range (pH 4–11) and exhibit broad substrate specificity. However, they have a lower reaction rate and worse heat tolerance than the bacterial enzymes (Pandey et al. 2000).

7.2.5 Amylases

Amylases are starch-degrading enzymes that catalyze starch by hydrolyzing internal glycosidic bonds in polysaccharides with the retention of anomeric configuration in products. Among starch-degrading enzymes are endo-amylases, exoamylases, debranching enzymes, and glycosyltransferases (Yadav et al. 2015; Khajeh et al. 2006). The enzymatic hydrolysis by amylases is preferred to acid hydrolysis in the starch processing industry due to the specificity of the reaction, the stability and lower energy requirements, and the elimination of neutralization steps (Satyanarayana et al. 2005). There is an increasing demand for amylases with better properties such as raw starch-degrading amylases, which are suitable for industrial applications and their cost-effective production techniques (Burhan et al. 2003). These enzymes account for about 30% of the world's enzyme production (Maarel et al. 2002). Most of the amylases are metalloenzymes which require calcium ions (Ca^{2+}) for their activity, structural integrity, and stability (Bordbar et al. 2005).

Most of the amylases have been produced from soil fungi such as *Aspergillus*, *Penicillium*, and *Rhizopus* (Pandey et al. 2000). Endophytic dark septate root endophytic fungi *Phialophora finlandia* and *P. fortinii* isolated from alpine plant communities were able to break down the major polymeric forms of carbon nitrogen and phosphorus found in plants (Caldwell et al. 2000). Similarly, raw starch-degrading enzyme is known to be produced from endophytic fungi *Gibberella pulicaris*, *Acremonium* sp., *Synnematous* sp., and *Nodulisporium* sp. (Marlida et al. 2000b). Maria et al. (2005) also reported the amylase production by few endophytic fungi isolated from mangrove plants *Acanthus ilicifolius* L. and *Acrostichum aureum* L. Sunitha et al. (2012) reported the amylolytic activity of endophytic *Cylindrocephalum* sp. on glucose yeast extract peptone agar medium. Endophytic *Cylindrocladium* sp. isolated from *Baccharis dracunculifolia* had produced the α-amylase and glucoamylase through fermentation in the rice-based solid state without supplementation (Onofre et al. 2011). Endophytic *Acremonium* sp. was able

to catalyze the hydrolysis of amylase and amylopectin. Glucose was the sole product indicating that this enzyme displays an extraction of starch-degrading activity (Marlida et al. 2000a).

7.2.6 Pectinases

Pectinase is a group of enzymes that break down pectin and depolymerize it by hydrolysis and by de-esterification reactions. Endopolygalacturonase, exo-polygalacturonase, exo-polygalacturonase, endopectate lyase, oligo-D-galactosiduronate lyase, and endopectinylase are depolymerizing enzymes that cleave glycosidic bonds of pectins by means of hydrolysis and transelimination (Alkorta et al. 1998). Pectin esterase is the pectolytic enzyme that catalyzes the hydrolysis of ester links between the carboxyl and methyl groups of complex polysaccharide known as pectin found in the cell wall of higher plants (Ceci and Lozano 1998). Pectic enzymes account for about 25% of world's food enzyme production (Kashyap et al. 2001). A variety of bacteria, yeasts, and molds are capable of producing pectic enzymes (Akhter et al. 2011). Many plant pathogenic bacteria and fungi are known to produce pectolytic enzymes essential for the decay of dead plant material by producing these enzymes and thus assist in recycling carbon compounds in the biosphere (Alkorta et al. 1998). Pectinases are frequently used in the fruit and vegetable industry and also employed widely in the textile and food industries (Alkorta et al. 1998). Pectinases are widely used in the wine industry for decreasing astringency by solubilizing anthocyanins without leaching out procyanidin polyphenols, and they also increase pigmentation by extracting more anthocyanins (Tucker and Woods 1991).

7.2.7 Ligninases

After cellulose, lignin is the second most abundant renewable biopolymer in nature. Lignin is an aromatic, three-dimensional, and amorphous biopolymer. It is synthesized from phenylpropanoid precursors by polymerization in higher plants; the lignin precursors p-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol consist of an aromatic ring and a three-carbon side chain (Brown 1985). In the lignin molecule, the precursors form three types of subunits: hydroxyl phenol (H type), guaiacyl (G type), and syringyl subunits (S-type). Lignin comprises 20–30% of woody tissue and forms a physical barrier that protects cellulose and hemicelluloses from degradative enzymes. Lignin, cellulose, and hemicellulose groups of enzyme activities have been shown to be important in ecosystem biodegradation (Raheem and Ali 2004). The major enzymes associated with the lignin-degrading ability are lignin peroxidase, manganese peroxidase, and laccases are secreted by filamentous fungi from the class basidiomycetes specifically white rot fungi which can to degrade the recalcitrant cell wall constituent lignin (Piontek et al. 2002; Wu et al. 2005).

Endophytic fungi such as *Alternaria*, *Phoma*, and *Phomopsis* isolated from *Colophospermum mopane* exhibited lignocellulolytic activity and degraded plant debris (Wang and Dai 2011). Chilean wood-inhabiting fungal endophytes *Bjerkandera* sp. and *Mycelia sterilia* of *Drimys winteri* and *an* unidentified basidiomycete and also basidiomycete *M. sterilia* of *Prumnopitys andina* were able to develop a nonselective white rot wood decay (Oses et al. 2006).

7.2.8 Lipases

Lipases are the hydrolytic enzymes classified as a special class of esterases that in vivo break the ester bond of triacylglycerol releasing free acids and glycerol (Oliveira et al. 2012). These are able to catalyze interesterification, alcoholysis, acidolysis, esterification, and aminolysis reactions in nature and when under proper conditions in vitro (Diaz et al. 2006). Endophytes able to produce lipases have been the target of research in the last years. An endophytic strain *Rhizopus oryzae* isolated from Mediterranean plants was able to catalyze the esterification of fatty acids in isooctane (Torres et al. 2003). Recently, an effort has been successful in the production and stabilization of lipase from endophytic *Cercospora kikuchii* (Costa-Silva et al. 2014). The endophytic yeast was able to produce lipase under submerged fermentation in a medium containing soybean oil as the main nutrient (Oliveira et al. 2012). In a study, endophytic *Fusarium oxysporum* isolated from the leaves of *Croton oblongifolius* Roxb. showed lipase activity on different medium with a wide range of pH (pH 8–12) (Corrêa et al. 2014). It was shown that partially purified enzyme containing lipolytic activity, produced by submerged fermentation in a lower-cost cultivation medium by endophytic yeast *Candida guilliermondii*, can be used as a catalyst for the production of methyl oleate using methanol as substrate (Oliveira et al. 2014)

7.2.9 Chitinases

Chitin, a linear homopolymer of β-1,4-linked N acetyl glucosamine, is a constituent of the exoskeleton of insects and shells of crustaceans and forms the basic structural component of the fungal cell wall. Chitinases are the enzymes that degrade this insoluble polymer also known as chitinolytic enzymes. Fungal chitinases play an important role in the ecosystem by degrading and cycling of carbon and nitrogen materials in chitin (Verma et al. 2015a, b, c, 2016a; Kellner et al. 2009). Chitinases of fungi are also being studied for their potential in biocontrol of nematodes (Gan et al. 2007) and pathogenic fungi (Klemsdal et al. 2006). Plants also produce chitinases as a defense response to infection by pathogens (El Gueddari et al. 2002). The products of chitinases have many desirable properties and find use in the control of microbes, tumors, wound healing, wastewater treatment, and drug delivery (Dai

et al. 2010). Chitinases have been reported from the endophytic fungi *Neotyphodium* sp. and *Colletotrichum musae* (Borges et al. 2009). It has been reported that the same endophytic fungi isolated from different host species showed the varied capacity to produce enzymes, for example, *Colletotrichum acutatum*, *Fusarium* sp., *Phomopsis* sp., and *Phyllosticta capitalensis* isolated from different plant hosts varied in their ability to produce the different chitin-modifying enzymes (El Gueddari et al. 2002).

7.3 Conclusion and Future Prospects

Endophytic fungi are unexplored source of novel secondary metabolites. The ability of production of extracellular enzymes from endophytic fungi provides wide scope for the production of industrial-based biocatalysts. New methods of cultivation and optimization are necessary to produce new and novel value-added products of interest in agriculture, medicine, paper and pulp, and many other industries. The products produced from unique biochemical pathways from fungi include many therapeutic compounds like anticancer compounds, antibiotics, immunosuppressants, antihyperlipidemics, and toxins. Genome-wide transcription profiling, proteomics, and reconstruction of complete metabolic will provide a better understanding of cellular processes and leads to the production of novel enzymes at industrial scale. The fungal genomic era has shown great and unexpected fungal ability for the biosynthesis of natural products. Genomic fungal sequences have revealed important information about novel gene clusters; however, collaborative work among chemists, mycologists, and geneticists is essential for better correlating the genomic information to the secondary metabolites and their functions. Recently, endophytic fungi are gaining much more attention for their biotransformation ability. The enzymatic biotransformed products obtained from endophytic fungi could be used as hits for drug design with therapeutic applications.

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Chapter 8
Biosynthesis of Fungal Chitinolytic Enzymes and Their Potent Biotechnological Appliances

Suman Kumar Halder, Shilpee Pal, and Keshab Chandra Mondal

Abstract Chitin is the world's second most abundant polysaccharide (after cellulose) and most plenteous amino-polysaccharide in environment. Its recalcitrant structure contributes mechanical strength to the chitin-bearing organisms. Chitinolytic enzymes or chitinases are group of glycosyl hydrolases which collectively and ultimately breaks chitin to its building block N-acetylglucosamine. Chitinolytic enzymes are ubiquitous among most of the living taxa, starting from bacteria to human beings, where they play different imperative biological functions. In spite of its cosmopolitan distribution in nature, chitinase from microorganisms are extensively explored. Chitinase has engrossed worldwide colossal attention due to its widespread applicability in biocontrol, biomedical, waste management, and pharmaceutical sectors, and owing to these employments, there is a steady increment in the demand of chitinases in present scenario. Perusal of literature attested that among the reports on microbial chitinase, a fungus contributes a lion's share. In fungi, chitinase plays multiple physiological roles including degradation of indigenous and exogenous chitin. Classical fermentation method in optimized condition is generally applied for the production of chitinase, whereas with the advent and advancement of genetic engineering, overproduction/overexpression of chitinase is now becoming a fascinated approach. In the present deliberation, biosynthesis of fungal chitinolytic enzymes, their classification, physiological role, potential applications, and future perspectives are outlined and highlighted.

8.1 Introduction

Enzymes are pervasive in all living taxa, of which numerous microorganisms from diverse exotic environments are found to be great producers of extracellular enzymes. With the progression of science and technology, the role of enzymes in biological

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processes has been explored. Fungal cell wall confers mechanical stability during their polar growth and cell division. In the last two decades, structural elucidation of fungal cell wall architecture enabled us to identify the array of enzymes associated with polysaccharide synthesis and remodeling. The principal component of fungal cell walls is chitin and is an essential scaffold made by homopolymerization of N-acetylglucosamine. Chitin is the most plenteous cationic amino-polysaccharide in biosphere, and its rigid and resistant structure contributes mechanical strength to the chitin-bearing organisms. For mycelial growth, continuous remodeling of fungal cell wall chitin is performed by an array of enzymes(often called chitinolytic system) with their synergistic and consecutive action, which hydrolyzes chitin into N-acetyl-β-D-glucosamine with chitooligosaccharides intermediates (Gortari and Hours 2008). As housekeeping gene products, chitinolytic enzymes act more specifically during mycelial growth, cell separation, chitinous nutrient assimilation, or competition with other fungi (Langner and Göhre 2016).The number of chitinase encoded genes in fungi varied species to species and also depends on habitat and niche. It was documented that filamentous fungi may encoded different types of chitinase gene (Rathore and Gupta 2015).

Besides fungi, chitinases are produced by varieties of organisms, from bacteria to human, irrespective of the existence or nonexistence of chitin in that particular organism and even fossils (Gortari and Hours 2008; Yadav 2015; Yadav 2017; Yadav et al. 2016a, b). In these organisms, they play diversified and important roles in nutrition, morphogenesis parasitism, struggle for existence, immunity, etc., which are sometimes obligatory for their survival (Fig. 8.1). Despite its abundance, no substantial accumulation of chitin in biosphere was noticed due to the action of chitinases in various organisms especially microorganisms. Microorganisms play a fundamental role in chitin cycling, and owing to this they are considered as natural resource of chitinolytic enzymes (Gortari and Hours 2008).

During 2010–2011, Indian biotech market grew at 21.5% to reach Rs. 17,400 crores in revenues, but unfortunately minimally share in the international market of industrially viable enzyme, which is projected to be US$ 3387.30 million (Binod et al. 2013; Halder et al. 2016). To compete internationally, we have to focus on the cost-effective production of industrially viable enzymes by valorizing renewable bioresources (Halder et al. 2016). Chitinases have imperative biophysiological functions and several potential applications and can be produced economically from chitin-rich low-cost seafood waste by microorganisms. Among all the chitinase-producing genera, bacterial chitinases are extensively explored and chatted. Recently, researches on chitinases of fungal origin have made rapid progress. Perusal of literature advocated that fungal chitinases are less extensively appraised in comparison to the bacterial chitinase, in spite of their much notable applicability. Considering this, the aim of the deliberation is to review and compile research work on biosynthesis of fungal chitinases, classification, and their potential applications.

Bacteria
- Mineralization of chitin, nutrition
- Cell wall synthesis, nitrogen metabolism
- Parasitism, competition with fungi
- Quorum-sensing in few pathogens

Plants
- Defense against fungal and bacterial pathogens (PR protein)
- some show ice structuring activity and providing cold or freezing tolerance
- Counteract oxidative stress, act as calcium storage proteins

Protozoa
- Perforation in the chitin containing peritrophic matrix of the mosquito midgut

Fungi
- Cell division, hyphal branching, differentiation, autolysis, morphogenesis
- Nutritional role in entomopathogenic and in mycoparasitic fungi
- Inhibition of growth of competitive fungi

Insects
- Cuticle degradation at different larval stages (ecdysis)
- Defensive role against own parasites

Animals and Mammals
- Defense against chitin-containing pathogens
- Acidic mammalian chitinase acts in innate immunity
- Associated with lysosomal lipid storage disorders, sarcoidosis, thalassemia, asthma

PHYSIOLOGICAL ROLE OF CHITINASE IN DIFFERENT PHYLA

CLASSIFICATION OF CHITINASE

Endochitinases (EC 3.2.1.14)

Cleaves chitin arbitrarily at internal β-1,4 glycosidic bond resulting in generation of water soluble, low molecular mass oligomers of N-acetylglucosamine (GlcNAc) named chitooligosaccharides (e.g. chitotetraose, chitotriose)

Exochitinase

Chitobiosidases (EC 3.2. 1.29) catalyzes the successive release of diacetylchitobiose from the non-reducing termini of chitin

β-N-acetyl glucosaminidases EC3.2.1.30) split diacetylchitobiose, chitotriose and chitotetraose, into GlcNAc

Fig. 8.1 Physiological role of chitinase among different taxa partially adapted from Gohel et al. (2006) with modification, and broader classification of chitinases on the basis of their pattern of cleavage of chitin (Gortari and Hours 2008; Halder and Mondal 2018, Halder 2018)

8.2 Classification, Catalytic Specificity, and Family of Fungal Chitinase

Nomenclature of chitinolytic enzymes is confused, and in this article, we theorized that chitinases are defined as any enzyme that catalyzes the cleavage of chitin (Duo-Chuan 2006). As per this nomenclature, the enzymes of the chitinolytic system are classified as endochitinases and exochitinases. Endochitinases (EC3.2.1.14) cleave chitin arbitrarily at internal glycosidic bond resulting in generation of water-soluble, low molecular mass oligomers of N-acetylglucosamine named chitooligosaccharides, such as chitotetraose and chitotriose, and ultimately giving diacetylchitobiose as predominant products. Exochitinase is subdivided into two categories: chitobiosidases and β-(1,4)-N-acetyl-glucosaminidases. Chitobiosidases (EC3.2.1.29) or chitin-1,4-β-chitobiosidases catalyze the successive release of diacetylchitobiose from the nonreducing termini of chitin fiber. β-(1,4)-N-acetylglucosaminidases (GlcNAcase, EC3.2.1.30) or chitobiases split diacetylchitobiose, chitotriose, and chitotetraose into GlcNAc through exo-pattern (Gortari and Hours 2008; Halder

and Mondal 2018; Halder 2018) (Fig. 8.1). The number of chitinases coding gene in fungi varied greatly and ranges from only 1 in *Schizosaccharomyces pombe* to greater than 30 in mycoparasitic *Trichoderma* spp. (Langner and Göhre 2016). However, most of the chitinolytic fungi have been found to produce more than one chitinase.

Chitinases are belongs to glycosyl hydrolase (GH) families, which are classified on the basis of sequence homology, and the up-to-date list of GH families is available through the CAZy database (http://www.cazy.org) (Cantarel et al. 2009; Adrangi and Faramarzi 2013). There are plentiful chitinases of fungal origin that were already available in database (e.g., NCBI). In silico analyses of the catalytic domains of members of each GH family fold into a common three-dimensional structure. As mentioned earlier, chitinolytic enzymes are categorized into two subgroups: chitinases that cleave the chitin chain internally and randomly belong to GH18, GH19, GH23 and GH48, and β-N-acetylhexosaminidases (β-N-acetylglucosaminidases) that catalyze the sequential removal of GlcNAc residues from the nonreducing end of the chain and belong to GH3, GH18, GH20, and GH84 (Adrangi and Faramarzi 2013). Among the aforementioned GH family, GH18, 20 and 84 have similar $(\beta/\alpha)_8$ barrel domains, while GH19 and 23 enzymes adopt an $\alpha + \beta$ structure. On the other hand, the catalytic domain of GH3 forms a bipartite structure, comprising $(\beta/\alpha)_8$ barrel followed by $(\alpha/\beta)_6$ sandwich. Besides, GH48 enzymes have an $(\alpha/\alpha)_6$ barrel structure characterized by six central and six external α-helices (Yoshida et al. 2010; Adrangi and Faramarzi 2013). A pool of genes encoding chitinases has been sequenced from different fungal strains. Recently, whole genome sequencing of many model fungi revealed the occurrence of multiple chitinases as well as variation in chitinase-coding gene. Fungal chitinases belong to GH18 family which is subgrouped into chitinases A, B, and C with respect to sequence and structural similarities and distinctive features (Seidl 2008; Hartl et al. 2012) (Figs. 8.2 and 8.3). One of the structural features of most chitinases is multidomain architecture, containing auxiliary domains such as the carbohydrate-binding modules (CBMs) in addition to a catalytic domain, for example, lysine motifs (LysM) (Fig. 8.3).

8.3 Indigenous and Exogenous Role of Fungal Chitinases

Published literature review advocates that role of chitinases in fungi is broadly bipartite: in growth and development, i.e., indigenous chitin degradation and exogenous chitin assimilation (Seidl 2008). During developmental and morphogenesis, issues like sporulation, spore germination, elongation, autolysis of hypha, cell wall remodeling, branching, etc. and also in cell division breakage within and in between chitin polymer were performed by chitinases. Chitinases of yeast are specially takes parts in budding. On the contrary, apart from the aforementioned role, several chitinase encoded genes of filamentous fungi take part in assimilation of exogenous chitin as nutrient. In symbiotic mycorrhizal association, the secretory chitinases of the fungi along with other hydrolyzing enzymes degrade

Fig. 8.2 A cladogram showing the evolutionary relationship of selected fungal chitinases from all genera represented in the NCBI database. The evolutionary history was inferred using the neighbor-joining method (Saitou and Nei 1987). The evolutionary distances were computed using the Poisson correction method and are in the units of the number of amino acid substitutions per site. The analysis involved 57 amino acid sequences. All positions containing gaps and missing data were eliminated. Evolutionary analyses were conducted in MEGA7 (Kumar et al. 2016)

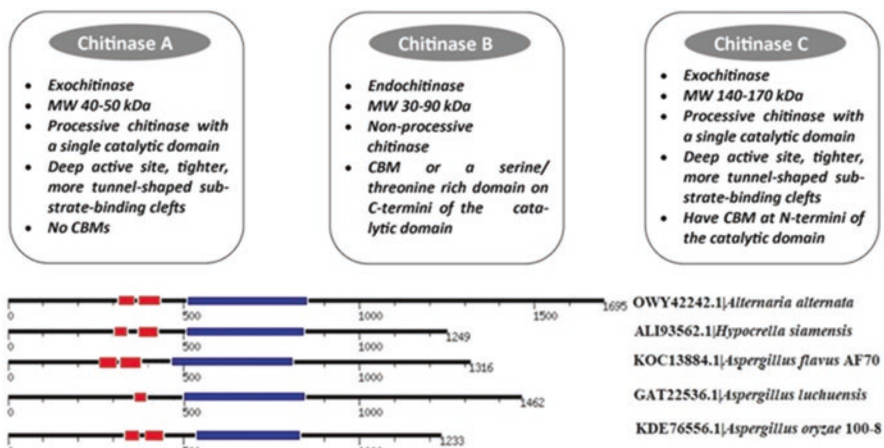

Fig. 8.3 The upper parts represent salient features of chitinases A, B, and C. The lower part represents alignment of five representative fungal chitinase protein sequences. Two domains, namely, glycosyl hydrolases family 18 [blue colored] and LysM (lysin motif) [red colored], are conserved in all cases

the organic residues in the rhizosphere into simpler form which is readily absorbed by plants. Endophytic fungi are generally found associative condition with their host plants where their secreted chitinases takes part to control the infection caused by chitin-bearing pests like plant-pathogenic fungi and nematodes (Matsumoto 2006). These types of differential physiological activity need spatiotemporal regulation of the chitinase activity (Langner and Göhre 2016).

8.4 Production of Fungal Chitinase

In biotechnological and industrial perspectives, filamentous fungi are well suited in industrial processes. Though many successful attempts were made for the production of chitinases from many filamentous fungi, *Trichoderma harzianum* has been commercialized for chitinase production in industrial set up (Gortari and Hours 2008). The microbial chitinases especially of fungal origin have grabbed wide attention in the biotransformation process to recycling of chitinous crustacean and shellfish shell waste (Dahiya et al. 2006; He et al. 2006; Rattanakit et al. 2002, 2008; Gortari and Hours 2008). Though bacterial chitinases have been reported to produce through submerged fermentation (batch, fed-batch, and continuous) extensively, solid-state fermentation is commonly adopted for the production chitinase by filamentous fungi. Generally, expression of microbial chitinases is inducible in nature (Gortari and Hours 2008; Halder et al. 2016), and therefore proper conditions should be maintained for getting maximal production of the enzyme.

Extracellular chitinase production is influenced by various physicochemical factors like carbon and nitrogen sources, salts, aeration, medium pH, fermentation temperature, moisture content, and inoculum volume (Sahay et al. 2017; Saxena et al. 2016; Suman et al. 2015; Yadav et al. 2012; Gortari and Hours 2008; Halder et al. 2013b). Optimization of these factors is executed by classical one variable at a time or statistical (response surface methodology) approach (Halder et al. 2013b). For improvement of chitinase production co-culture, biphasic culture and cell immobilization have also been reported (Gortari and Hours 2008). Even though wild-type organisms are the major bioreactor for chitinase biosynthesis, genetic improvement of the wild-type organisms may enhance their efficiency and potential. With the advent and advancement of genetic engineering, traditional strain improvement by random or site-directed mutagenesis is replaced by transgenesis. Recombinant DNA technology displays a wide range of possibilities few of which will be discussed in the subsequent section.

8.5 Application of Chitinase and Chitinolytic Microbes

Since the last two decade, microbial chitinases have grabbed wide attention owing to their wide range of appliances. Besides imperative physiological tasks, fungal chitinases have been publicized for their immense potential application in diversified fields. So far, a number of chitinases produced by different fungal strains were reported which shows expanded properties and multitude of applicabilities. In Fig. 8.4, outlines of well-established application fungal chitinases have been depicted. In the following section, real-world/practical applications of the chitinases and how they could be helpful to industry and human affair for maintaining the steady state in the nature and mankind were highlighted.

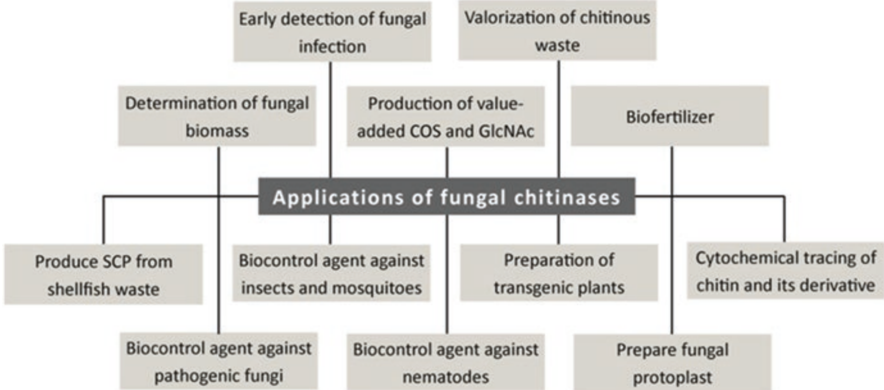

Fig. 8.4 Well-known applications of fungal chitinases

8.5.1 Production of Single-Cell Proteins (SCP) from Shellfish Waste

In recent years, constant increase in the exploitation of fish resources for human consumption is recognized. Seafood waste which is one of the major by-products of shellfish processing industries is a rich source of chitin and protein, and therefore this can be utilized for single-cell protein (SCP) production. In this approach chitinase digested chitinous waste can be utilized as carbon, nitrogen, and/or other nutritional source for production of microbial biomass. In this context, chitin hydrolysate prepared by the chitinolytic enzymes of *Myrothecium verrucaria* was used as a substrate for SCP production using *Saccharomyces* (Vyas and Deshpande 1991). Accordingly, chitinase-producing yeast *Pichia kudriavzevii* was also employed for SCP production (Revah-Moiseev and Carrod 1981). SCP production using *Penicillium ochrochloron* MTCC 517 chitinase was successfully achieved, and the same was supplemented during fish meal formulations. Results revealed that supplementation of SCP with diet imparted better growth response in fish *Lepidocephalus thermalis* (Patil and Jadhav 2014).

8.5.2 Biocontrol Agent Against Pathogenic Fungi, Insects, and Nematodes

Human beings are overwhelmed by pathogenic consequences and health-related issues. An array of fungi, insects, and nematodes are theorized as a major problematic biological agent as they are responsible for significant economic losses in agriculture and affecting public health. Due to worldwide rise against the application of chemical pesticides and their long-lasting adverse effects on human and health ecosystems, control of the aforementioned organisms through biological means becomes an alternative in modern agriculture for minimizing the constrains which also nullified the possibility of generation of resistant strains. Since chitins are a major and common constituent of fungi, insects, and nematodes (egg), they are readily susceptible to different chitinolytic enzymes. As mentioned earlier, chitinase belongs to the pathogenesis-related proteins (PR proteins) produced by plants in response to viral, bacterial, and fungal infection (Roopavathi et al. 2015). Besides bacteria and actinomycetes, fungi were reported to produce fungicidal chitinases. Chitinase-producing mycoparasitic fungi can be used in agriculture as an effective biocontrol agent against a number of phytopathogenic fungi (Table 8.1). Chitinase can be effectively control the common plant-pathogenic fungi, viz., *Alternaria*, *Aspergillus*, *Botrytis*, *Cladosporium*, *Fusarium*, *Monilinia*, *Penicillium*, and *Ramularia*, which attack various horticultural plants like vegetables, fruits, and ornamental flowers (Brzezinska et al. 2014).

Chitin is present in the exoskeleton and gut linings of insects. Peritrophic membrane (PM) is situated in most insects' midgut acts as a mechanical protective bar-

Table 8.1 Antifungal activity of few reported fungal chitinases

Fungal chitinase source	Target fungal species	References
Aspergillus niger	*Fusarium culmorum*, *Fusarium solani*, *Rhizoctonia solani*	Brzezinska and Jankiewicz (2012)
Aspergillus terrus	*Aspergillus niger*, *Aspergillus oryzae*, *Penicillium oxysporum*, *Rhizoctonia solani*	Firag and Al-Nusarie (2014)
Basidiobolus ranarum	*Rhizoctonia solani*, *F. solani*	Mishra et al. (2012)
Lecanicillium lecanii	*Fusarium oxysporum*, *Rhizoctonia solani*	Nguyen et al. (2015)
Myrothecium verrucaria	*Puccinia arachidis*	Govindsamy et al. (1998)
Penicillium janthinellum	*Mucor plumbeus*, *Cladosporium cladosporioides*	Giambattista et al. (2001)
Penicillium ochrochloron	*Fusarium oxysporum*	Patil et al. (2013)
Trichoderma atroviride	*Rhizoctonia solani*	Harighi et al. (2006)
Trichoderma harzianum	*Macrophomina phaseolina*, *Fusarium* sp., *Rhizoctonia solani*, *Aspergillus niger* (NCIM 563), *Aspergillus* sp., *Rhizopus* sp., *Mucor* sp.	Bell et al. (1982), Nampoothiri et al. (2004), Monteiro et al. (2010)
Trichoderma harzianum	*Fusarium oxysporum f. sp. melonis*, *Sclerotium rolfsii*	Viterbo et al. (2001)
Trichothecium roseum	*Alternaria alternata*, *Fusarium moniliforme*, *Magnaporthe grisea*	Duo-Chuan et al. (2004)

rier. Chitinase is therefore is a metabolic target of selective insect control agents. Chitinase is a virulence factor due to its ability to degrade the chitin content of exoskeleton and PM which leads to osmotic lysis and impairment of nutritional absorption in the midgut, respectively, which eventually leads to death of the insect. The chitinolytic system of entomopathogenic fungus *Metarhizium anisopliae* digested insect cuticles during diseases development (de Assis et al. 2010). Larvicidal potential of *Trichoderma harzianum* chitinase against the *Helicoverpa armigera* (cotton bollworm) was evaluated where the chitinase acts as potential antifeedant which reduces the feeding rate as well as larval body weight (Binod et al. 2007). Likewise, species belonging to *Lecanicillium* were reported to secrete chitinases which penetrate insect integument and considered as insect pathogens; few of them were commercially explored as biopesticide in agriculture (Goettel et al. 2008). It was reported that the insecticidal activity may be improved by the addition of adjuvants [e.g., polyoxyethylene-(3)-isotridecyl ether] which facilitate the efficacy of the enzymes through epicuticle (Adrangi and Faramarzi 2013).

Mosquitoes are the vectors of several illnesses like chikungunya, dengue, encephalitis, malaria, and yellow fever, which are associated with significant morbidity and mortality in humans and domestic animals, and hence are of nascent targets for biocontrol agents. Due to increased drug resistance of mosquitoes against

various chemical mosquitocidal agents, recently, chitinase is employed as mosquitocidal agent to combat with the diseases. Both first and fourth instar larvae of mosquito *Aedes aegypti* were killed within 48 h by treating with crude preparation of chitinases, proteinases, and lipases from *Myrothecium verrucaria* (Mendonsa et al. 1996). Chitinase of *Beauveria bassiana* was reported as effective larvicidal and pupicidal agents against *Aedes aegypti*, *Anopheles stephensi*, and *Culex quinquefasciatus* (Ragavendran et al. 2017).

Though nematode eggs are one of the most resistant biological structures, they are vulnerable to being attacked by egg-parasitic fungi (Gortari and Hours 2008). Nematophagous fungi secreted chitinase which specifically acts on the nematode egg as it is the only structural element where abundance of chitin has been documented. Chitinase acts with other cooperative enzymes like proteases, lipases, and lysozymes, and most of the studies related to nematophagous fungi so far have been restricted to plant parasites (Gortari and Hours 2008;Tikhonov et al. 2002). Ovicidal activity of fungal chitinase on *Ascaris lumbricoides* was reported by Kunert et al. (1985), whereas chitin breakdown that leads to premature hatching resulting in fewer alive juveniles was documented by Mercer et al. (1992). Nematocidal activity of purified chitinase of *Verticillium chlamydosporium* (syn. *P. chlamydosporia*) and *Verticillium suchlasporium* Zare and Gams (syn. *Pochonia rubescens*) was demonstrated by treating the purified chitinase in single or in combination with purified proteases on *Globodera pallida* egg (Tikhonov et al. 2002). In accordance with the above reports, treatment with both chitinase and protease of fungal origin was found promising against eggs of *Meloidogyne javanica*, *Caenorhabditis elegans*, and *Meloidogyne incognita* to reduce their population (Khan et al. 2004; Park et al. 2004; Gan et al. 2007). Nematode-pathogenic *Clonostachys rosea* was reported to produce some proteases and chitinases which are exploited as biocontrol agents to abate the propagation of both plant and animal parasitic nematodes (Adrangi and Faramarzi 2013).

8.5.3 Genetically Engineered Plants

Overexpression and engineering (genetic and metabolic) of the fungal chitinases could increase their efficiency as a biocontrol agent. Constant bioprospecting for novel, hyper-chitinolytic fungal strain for direct application as well as their genetic manipulation in developing potential biocontrol strategy is in progress. Such studies are indispensable for developing a more efficient chitinase producer and production of transgenic plants which can combat with fungal and insect pathogens. The possibility for improving plant resistance through genetic manipulation is currently the emerging area of research, and quite successful in increasing resistance to diseases caused by biotrophic and necrotrophic fungal pathogens. Engineered plants were successfully made by transfer of the antifungal endochitinase gene of *T. harzianum* in tobacco, apple, and potato, which was expressed constitutively and imparted enhanced resistance against tested phytopathogenic fungi (Duo-Chuan

2006). Likewise, transgenic tobacco expressing *S. cerevisiae* chitinase (Cts1) was inhibited both spore germination and hyphal extension of *Botrytis cinerea*. Studies in biochemical and molecular level for knowing detailed insight of the underlying mechanism of chitinase secretory process and in view of that development of cloning strategies for secretion of desired products may collectively lead to more disease-resistant transgenic plants in the near future.

8.5.4 Preparation of Fungal Protoplast

Fungal protoplasts are being used as an effective experimental tool in studying cell wall synthesis, enzyme synthesis and secretion, in monitoring the effects of toxicants, as well as for inserting desirable genetic trait for strain improvement (Rathore and Gupta 2015). The major application of chitinases is the dissolution of chitin-containing cell wall of fungi to accelerate protoplast generation. Release of protoplasts from *Schizophyllum commune* by mutual action of purified chitinase and α-1,3-glucanase of *Trichoderma viride* was documented by de Vries and Wessels (1973). In another instance, chitinase of *Penicillium ochrochloron* MTCC 517 in combination with β-glucuronidase and lysing enzyme effectively generated protoplast of *Aspergillus sojae* NCIM 1198, *Trichoderma harzianum* NCIM 1185, *Aspergillus oryzae* NCIM 1272, *Rhizopus oligosporus* NCIM 1215, and *Neurospora crassa* NCIM 870 (Patil and Jadhav 2015).

8.5.5 Tracing Cytochemical Localization of Chitin, Chitosan, and Chitooligomers

Chitin is the major biopolymer of fungal cell wall. Besides biochemical analysis, a cellular localization study is essential to understand the functional specialization of these polymers. Localization of chitin in the cells of tomato root infected by *Fusarium oxysporum* f. sp. *radicis-lycopersici* was detected through chitinase-gold probe (Chamberland et al. 1985). Benhamou and Asselin (1989) used wheat germ agglutinin ovomucoid-gold complex and chitinase-gold complex as probes for detection of GlcNAc residues in the secondary cell wall of plants, and both of them were found promising. Gold-labeled chitinase was also used by Araujo et al. (1993) for the detection of chitin in the cuticle of microfilariae of *Wuchereria bancrofti*. Chitinase-gold-labeled complexes have also been used for the localization of chitin and N-acetyl-D-glucosamine residues in a biotrophic mycoparasite, *Piptocephalis virginiana* (Manocha and Zhonghua 1997). Though recent studies in this aspects are limited due to development of innovative tools for cell biology study, this approach is still promising.

8.5.6 Determination of Fungal Biomass and Early Detection of Fungal Infection

There is a positive correlation between fungal growth in soil and activity of chitinolytic enzymes. As the chitinolytic activity has cosmopolitan distribution among fungal species, estimation of the former could be an indirect and quick measurement of fungal biomass (population) in soil or any sample. Assaying of N-acetylglucosaminidase activity provides a simple, sensitive, and fast (fluorogenic) measure of soil fungal biomass. Invasive fungal infections are one of the greatest causes of death of immunosuppressed patients due to the fact that the available diagnostic methods of detection are usually not responsive in the early state of infection. In this context, chitinase can be used for detection the same early and the method may be radioactive isotope or immunofluorescence based. However, extensive studies are required for clinical use of those techniques.

8.5.7 Production of Value-Added Chitinolytic Products and Valorization of Chitinous Waste

Chitooligosaccharide (COS), chitobiose, N-acetylglucosamine, and glucosamine have immense pharmaceutical relevance because of their broad range of agricultural, medical, and industrial applications as antibacterial, antifungal, hypocholesterolemic, anticancer, antitumor, antioxidant, antihypertensive, and food quality-enhancing agent (Halder et al. 2013a, b, 2014; Halder and Mondal 2018). Chitooligosaccharides of specific size can be obtained from the partial hydrolysis of chitin and chitosan. Specific blends and ratio of chitinolytic enzymes are desirable to get the desired chain length of the chitin/chitosan oligomer. For instance, admixture of high amount of endochitinase and low amount of N-acetylglucosaminidase and exochitinase leads to the production of chitooligosaccharides, whereas reversion of the quantity leads to the generation of GlcNAc (Dahiya et al. 2006). Chit42 of *Trichoderma harzianum* which hydrolyses chitin into its oligomers was expressed in *Pichia pastoris* (Kidibule et al. 2018). Seafood-processing industries all over the world generated a huge amount of chitinous biowaste, and disposal of this biomaterial created environmental pollution and related issues (Thadathil and Velappan 2014). Chitinase-producing microorganisms (especially bacteria and few fungi) are found promising for bioconversion of marine crustacean biomaterials and concomitant production of enzymes and/or bioactive chitooligosaccharides, which in turn made the process favorable in terms of commerce and environment. *Aspergillus* sp. S1-13 utilizes shrimp shellfish waste as substrate for solid-state production of chitinases, which subsequently established their role in chitin-saccharification (Rattanakit et al. 2002, 2008).

8.5.8 Biofertilizing Activity of Chitinase and Chitinolytic Products

As mentioned earlier, chitinases have immense biopesticidal potentiality which attested their application in plant growth promotion. Moreover chitooligosaccharides also have wide scopes of application in agriculture owing to their eliciting activities in plants against microbial infections leading to a variety of defense responses which in turn maximize yield and quality of crops (Halder and Mondal 2018). Beside the use of pure chitooligosaccharides as fertilizer, chitinous waste materials could be used as fertilizer as the chitin materials are degraded by indigenous microorganisms upon application. Moreover, the fermented by-products after solid-state and liquid-state production of chitinase may contain reasonable amount of chitooligosaccharides which may be applied as fertilizer. In this context various attempts are executed to validate the use of either chitin-rich waste or its fermented by-products as biofertilizer. Biofertilizing efficiency chitinous waste was tested by mixing it (at varies concentration) with *Triticum durum* (wheat) soil (Kour et al. 2017).

It was found that soil microbial ecology and pH greatly changed which favors plant growth. During the initial 10 days after application, *Bacillus* sp. becomes predominant with no detectable growth of saprophytic or phytopathogenic fungi, whereas in the last 10 days, dynamics of rhizospheric microflora especially plant growth-promoting rhizobacteria (PGPR) was increased with the increase of load of nitrogen-fixing bacteria (Aïzi and Cheba 2015). It can be postulated that the chitin materials are degraded into its oligomeric form by the indigenous chitinolytic microbes present that habitat which helps plant and other bacteria for their growth. On the contrary, lipo-chitooligosaccharide (lipo-COS) is a potential nodulation factor, and foliar application of the same to tomato (*Lycopersicon esculentum*) boosts early flowering and increased fruit yield (Chen et al. 2007). Few patents are available which attested the fertilizing activity of COS and lipo-COS. Sahu et al. (2017) reviewed applications of shellfish shell waste-derived chitosan and COS in horticulture and agriculture. Mutifunctional role of seafood shell-derived chitosan in horticultural crops is documented by Sharif et al. (2018).

8.5.9 Miscellaneous Application Potential

Chitinase has a prospective to use as antifungal cream and lotion (Hamid et al. 2013). Owing to its antifungal activity direct medical use of fungal chitinase has been proposed (Pope and Davis 1979; Orunsi and Trinci 1985). The chitinase producers appear potential candidates for enhancing shelf life of various foods by controlling fungal infections (Hassas-Roudsari and Goff 2012). In medical field, chitinase is used as biomarker of many diseases, like acidic mammalian chitinase for asthma and chitotriosidase for microfilarial infection (Adrangi and Faramarzi

2013). Cody et al. (1990) suggested the enzymatic conversion of chitin to ethanol by *Pachysolen tannophilus* and *Zymomonas mobilis*. Direct ethanol production from N-acetylglucosamine and chitin substrates by *Mucor* species was also reported by Inokuma et al. (2013). Chitinase of endophytic fungal origin needed to be addressed in future which may open new dimension (Rana et al. 2016a, b, 2017).

8.6 Conclusion and Future Prospect

Since the day of discovery, research on the enzyme acts on the world's second most abundant biopolymer are carried out, but still scope for further research in different fundamental aspects of applications of chitinase is open. Recent development in molecular biology tools and techniques enabled us to sequence many new chitinase genes, and mining of the retrieved data gives us innovative information about the complexity and variety of chitinase that was not previously anticipated. The potential roles of fungal chitinases in exogenous chitin degradation made it promising candidate in biocontrol sector, waste valorization, SCP production, protoplast preparation, value-added COS and GlcNAc production, and so many other notable applications. Genetic and metabolic engineering of chitinase-producing organisms may lead to next generation of biofuel production from chitinous biowaste. We are optimistic that rapid development of the omics tools and techniques will address many unsolved twists and questions which may open a new vista in chitinase research.

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Chapter 9
Proteases from Extremophilic Fungi: A Tool for White Biotechnology

Richa Salwan and Vivek Sharma

Abstract Proteases are enzymes that degrade proteinaceous materials and find applications in detergents, leather, food, agriculture, pharmaceuticals, and bioremediation. They are produced by plants, animals, fungi, and bacteria. Among all, fungi produce acidic, neutral, and alkaline proteases, whereas bacteria produce only alkaline and neutral proteases. Despite the availability of microbial proteases in huge amounts, less number of proteases has been commercialized due to high cost and less stability to withstand harsh conditions in industrial processes. To meet the industrial demand, proteases have been engineered using genomic tools including recombinant DNA technology, site-directed mutagenesis, codon optimization, and nucleotide shuffling for enhanced expression. On the other hand, fungi living in extreme habitats have gained considerable importance for producing efficient proteases which can easily withstand conditions applied in industrial processes. Moreover, the downstream processing and recovery of fungal proteases is easy and cost-effective which is a major obstacle in industrial processes. Therefore, fungal proteases have high industrial demand due to stability and catalytic activity, broad diversity, and substrate specificity required in various bioengineering and biotechnological applications. This chapter illustrates type of proteases and their sources, characteristic properties, and their engineering for various biotechnological applications.

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9.1 Introduction

Proteases are hydrolytic enzymes and have attained central importance in industrial applications which involves selective degradation of proteins. The present cost for the sale of industrial enzymes is about $8 billion of which 65% is contributed by proteases alone (Barzkar et al. 2018; Omrane Benmrad et al. 2016). The specific hydrolytic behavior of proteases imparts applications in leather, food, pharmaceutical, detergent, silk-degumming, silver recovery, waste management, and peptide synthesis (Abidi et al. 2011; Gupta et al. 2002; Johnvesly and Naik 2001; Li et al. 2013; Rao et al. 1998; Savitha et al. 2011). Among the proteases produced by plants, animals, and microbes, about 40% of the total sale is contributed by microbes alone. The microbial proteases serve as preferred driver of increasing economy in the area of white biotechnology because of their desired characteristics needed in the industrial processes. Bacteria produce majority of the proteases as neutral and alkaline in nature, but proteases from fungi are neutral, acidic, or alkaline in nature, and ~60% of these have been commercialized till 2009 (Inacio et al. 2015). Various neutral proteases such as Umamizyme have been utilized in food industry due to their specificity in breaking hydrophobic bonds at neutral pH (Sandhya et al. 2005).

Fungal proteases have high industrial demand due to high stability and catalytic activity, broad diversity, and substrate specificity required for various bioengineering and biotechnological applications. Moreover, the extracellular enzyme production from fungal species leads to cost-effective production because of easy downstream processing and recovery of enzymes which is a major obstacle in industrial processes (Nakamura et al. 2011; Shaba and Baba 2012). Attention has been paid to microbes residing in hypersaline habitats (Gaba et al. 2017; Saxena et al. 2016), high pressures, extreme low temperature (Yadav 2015; Yadav et al. 2015a, b, c, 2016), and high temperature (Kumar et al. 2014; Sahay et al. 2017; Suman et al. 2015; Verma et al. 2015) which are able to produce enzymes with unique properties to act under extreme conditions (Dalmaso et al. 2015). Accordingly, extracellular proteases have been reported from psychrophilic *Glaciozyma antarctica*, *Candida humicola*, and *Rhodotorula mucilaginosa*; mesophilic *Aspergillus niger* and *Trichoderma harzianum*; and thermophilic *Thermomyces lanuginosus*, *Penicillium duponti*, and *Sporotrichum thermophile* (Duarte et al. 2018). Limited studies have reported expression of proteases from extremophilic fungi *Thermomonospora fusca* YX, *Chaetomium thermophilum* (Kim and Lei 2005; Li and Li 2009), *Aspergillus niger* (Katsuya et al. 1993; Pel et al. 2007), *Chaetomium thermophilum* (Li and Li 2009), *Fusarium oxysporum* (Di Pietro et al. 2001), *Penicillium oxalicum* (Shen et al. 2001), and *Trichoderma harzianum* (Liu and Yang 2007) in heterologous host for enhanced production to meet the industrial demand.

9.2 Sources of Proteases

Proteases play an important role in physiological processes like growth and differentiation, metabolic processes, gene expression, and cell signaling (Banerjee and Ray 2017; Sharma et al. 2016). Although proteases are produced by plants and animals, microbes are considered as promising candidates because of their diverse biochemical properties, limited space requirement, and ease of genetic modifications (Rao et al. 1998). Among microbes, fungi are generally preferred over bacteria as they are considered as safe candidates and produce extracellular proteins with high yields (de Souza et al. 2015; Hajji et al. 2010). Many filamentous fungal species including *Aspergillus*, *Chrysosporium*, *Fusarium*, *Penicillium*, *Pleurotus*, *Rhizopus*, *Scedosporium*, and *Trichoderma* are known to produce proteases (Kredics et al. 2005; de Souza et al. 2015; Inacio et al. 2015; Sharma et al. 2016; Banerjee and Ray 2017). Besides these, proteases from basidiomycetes such as *Agaricus bisporus*, *Armillariella mellea*, *Flammulina velutipes*, *Grifola frondosa*, *Pleurotus ostreatus*, *Pleurotus eryngii*, *Phanerochaete chrysosporium*, and *Schizophyllum commune* have also been reported for protease production (Inacio et al. 2015; Ellaiah et al. 2002; Sharma and De 2011; Novelli et al. 2016). The studies on proteases from fungi are increasing day by day, yet the full potential of all fungal classes has not been exploited for protease production (Sabotic et al. 2007). Majority of the proteases has been commercialized by submerged fermentation of fungi either in constitutive or inducible manner.

9.3 Proteases and Their Classification

Proteases are enzymes catalyzing the total breakdown of peptide bonds in proteins into oligo or amino acid units. They are assigned under group 3 as hydrolases and subgroup 4 for hydrolyzing peptide bonds according to International Union of Biochemistry and Molecular Biology (IUBMB) nomenclature. Further 11 to 24 classes of proteases are assigned. The general classification of proteases is based on reaction type, mode of action of proteases, and active site residue present (Rao et al. 1998; Dalmaso et al. 2015). Based on the type of reaction, these can be acid proteases which are active in pH range 2.0–3.5, neutral proteases active at pH 6.5–7.5, and alkaline proteases active at 9–10. On the basis of mode of action, proteases can be exopeptidases which break peptide bonds near N- or C-terminal of the substrate and endopeptidases which break peptide bonds away from the N- or C-termini (Fig. 9.1). Further, proteases are differentiated into serine, cysteine, aspartic, and metalloproteases based on functional group present in the active site (Fig. 9.1). Few proteases like glutamic acid and prolyl specific have also been reported in fungi (Sims et al. 2004; Tsiatsiani et al. 2017).

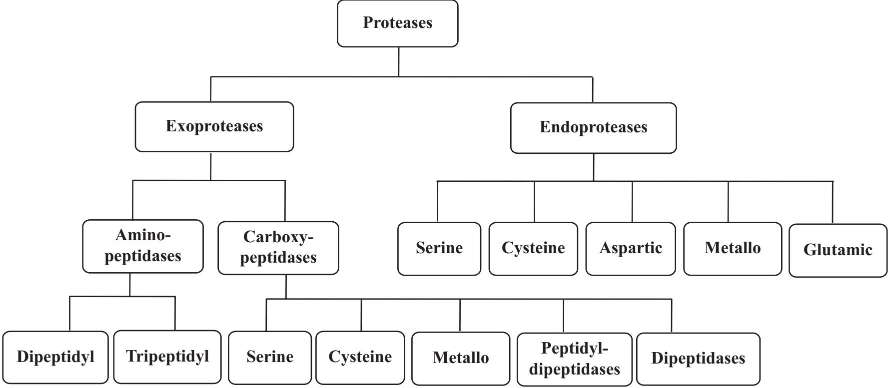

Fig. 9.1 Classification based on the mode and site of action of proteases. (After Rao et al. 1998)

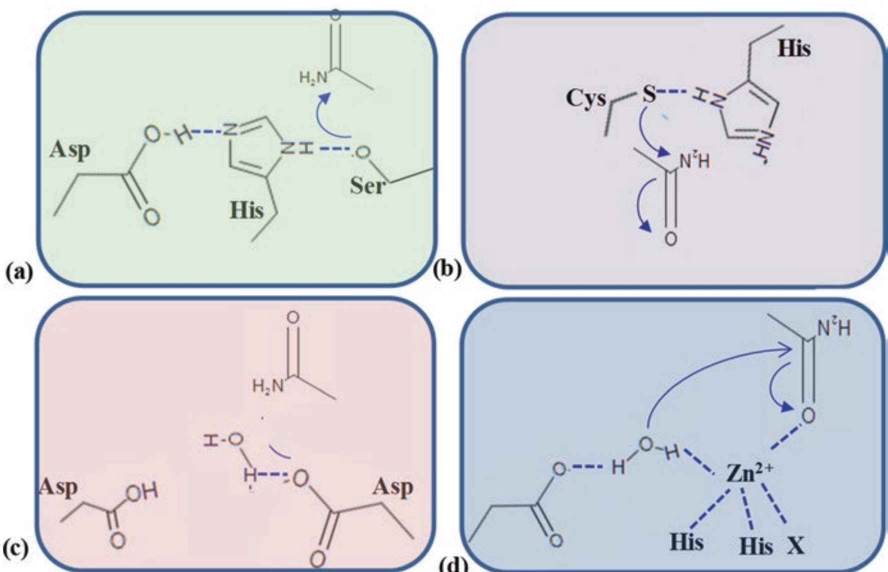

Fig. 9.2 Groups of proteases into (**a**) serine, (**b**) cysteine, (**c**) aspartic acid, and (**d**) metalloprotease based on the functional group present and mechanism of action. (After Erez et al. 2009)

9.3.1 Serine Proteases (EC 3.4.21)

Serine proteases have serine residue in their catalytic triad made of aspartate (D), histidine (H), and serine (S) (Fig. 9.2a). These proteases have conserved glycine residues near the catalytic serine which forms Gly-Xaa-Ser-Yaa-Gly as motif (Brenner 1988). Serine proteases can act at the amino or carboxy terminal or away from the polypeptide. The activity of serine proteases is strongly inhibited

in the presence of phenylmethylsulfonyl fluoride, thiol reagents, diisopropyl fluorophosphate, tosyl-L-lysine chloromethyl ketone, and 3,4-dichloroisocoumarin which breaks disulfide bonding of cysteine residues present near the active site.

9.3.2 Cysteine/Thiol Proteases (EC 3.4.22)

The cysteine proteases have catalytic triad made up of Cys-His-Asn which is similar to the Ser-His-Asp reported in serine-type proteases (Fig. 9.2b). These can be found in prokaryotes as well as eukaryotes and grouped into papain-like, trypsin-like, glutamic acid-specific, and others. The activity of cysteine proteases is inhibited in the presence of sulfhydryl agent *p*-chloromercuribenzoate, while DFP and metal-chelating agents have no influence. Cysteine proteases papain, clostripain, and streptopain are the most important types.

9.3.3 Aspartic Proteases (EC 3.4.23)

These proteases have two conserved aspartic acid residues situated in Asp-Xaa-Gly motif in their catalytic domain (Fig. 9.2c). These are generally active at acidic pH and hence known as acidic proteases. Microbial aspartic proteases are grouped into pepsin-like enzymes and rennin-like enzymes (Rao et al. 1998). The aspartic proteases are inhibited by pepstatin, 1,2-epoxy-3-(*p*-nitrophenoxy) propane (EPNP), and diazoacetyl-DL-norleucine methyl ester (DAN) in the presence of copper ions (Rao et al. 1998).

9.3.4 Glutamic Acid Proteases (EC 3.4.23)

These proteases contain glutamic acid and glutamine residues which form dyad in their active site. Previously considered as aspartate protease, some glutamic acid proteases contain glutamic acid and aspartate residues in their active sites. Since most active at pH 2, they are also called acidic proteases and are inhibited in the presence of 1,2-epoxy-3-(*p*-nitrophenoxy) propane (Murao et al. 1973).

9.3.5 Metalloproteases (EC 3.4.24)

Metalloproteases need divalent cations (Fig. 9.2d) and can be inactivated by the addition of chelating agents such as EDTA. They can also act as endopeptidases or as exopeptidases and include enzymes such as collagenases, matrix metalloproteases, hemorrhagic toxins, and thermolysin (Rao et al. 1998). Metalloproteases can be neutral, alkaline, Myxobacter I for small amino acid residues, and Myxobacter II

for lysine residues present in the peptide chain. Further based on the similarities in amino acid sequence, structure, and their evolutionary history, an online MEROPS database has been reported for the deposition of proteases information (Rawlings et al. 2008). Twelve versions of this database have been published till date. Over 9 lakhs protease sequences have been deposited, and the identifiers have been classified into 62 clans and 268 families (Rawlings et al. 2018). Over 1703 sequences have been successfully identified as serine type, and 171 sequences were assigned EC number (https://www.ebi.ac.uk/merops/cgi-bin/statistics_index?type=P).

9.4 Proteases from Extremophilic Fungi

The protease production from fungi thriving under extreme environmental conditions is a promising alternative for applications in industry. Such exotic microorganisms inhabiting hot waters, Arctic waters, and extremely saline, acidic, and alkaline environment are categorized into thermophiles, psychrophiles, halophiles, acidophiles, or alkaliphiles, respectively (Bertemont and Gerday 2011; Yadav et al. 2018). These microbes sustained their life by modifying their cellular and molecular constituents and produce enzymes with improved properties for suitability as additives in industrial processes.

9.4.1 Psychrophilic Fungi

Fungi can grow well at low temperature and inhabit permafrost (Golubev 1998), glacial ice (Ma et al. 1999), offshore polar waters (Broady and Weinstein 1998), glaciers, ice sheets, sea ice, icebergs, and freshwater ice (Salwan et al. 2010; Tojo and Newsham 2012). The fungi surviving at extreme low temperature are known as psychrophilic fungi due to their ability to tolerate cold environment. The optimum temperature for the growth of psychrophilic fungi is generally 10 °C but can even grow at lower temperature (Deverall 1968). Further, Morita stated that the fungi able to grow at 15 °C are known as psychrophilic and if fungi show its maximum growth at 20 °C are known as psychrotrophic fungi (Maheswari 2005; Hassan et al. 2016). Various fungal species belonging to the genera *Aspergillus ustus*, *Cryptococcus gilvescens*, *Humicola marvinii*, *H. fuscoatra*, *Mrakia gelida*, and *Rhodotorula laryngis* have been reported for proteases with application as detergent and textile additive (Damare et al. 2006a, b; Turchetti et al. 2008).

9.4.2 Thermophilic Fungi

The fungi that require 20 °C or above as their minimum growth temperature and 50 °C or above as their maximum growth temperature are known as thermophilic fungi. Some fungi that can tolerate temperature below 20 to 55 °C for their growth

are known as thermotolerant (Maheshwari et al. 2000). They generally grow on heaped masses of plant, agricultural, and forestry products where humidity, oxygen, and organic matter remain sufficient for their growth (Maheshwari et al. 2000). High temperature tolerance in fungi is not a common phenomenon as reported in bacteria which can tolerate temperature up to 100 °C (Brock 1995). Among fungi, only 30 species have been reported to tolerate 40–45°C among 50,000 recorded species. *Talaromyces thermophilus*, *Thermoascus aurantiacus*, *Thermomyces ibadanensis*, and *T. lanuginosus* have optimum temperature 42–52 °C but can tolerate up to 61 °C for growth (Maheshwari et al. 2000). Therefore, attention is paid to explore temperature tolerance attribute of fungi which has been less explored comparatively. These thermophilic fungi are able to produce extracellular proteases and reduce microbial contamination from other organisms during the protease production (Chen et al. 2004). Limited studies on proteases active within pH 3–6 and 45–55 °C have been done on thermophilic fungi *Mucor pusillus*, *Penicillium duponti*, *Malbranchea pulchella* var. sulfurea, and *Humicola lanuginose*. Proteases from thermophilic fungi serve as promising candidates for industrial applications due to their high specific activity and stability. Gene encoding protease from thermophilic *Chaetomium thermophilum* has been introduced in *P. pastoris* (Kim and Lei 2005; Li and Li 2009).

9.4.3 Mesophilic Fungi

Fungi able to grow at moderate temperature range 20–30 °C are known as mesophilic fungi. The genes encoding extracellular proteases have been characterized from *Aspergillus niger* (Gomi et al. 1993; Pel et al. 2007), *Aspergillus fumigatus* (Vickers et al. 2007), *Chrysosporium keratinophilum* (Dozie et al. 1994), *Neurospora crassa* (Abbott and Marzluf 1984), *Penicillium oxalicum* (Shen et al. 2001), and *Trichoderma harzianum* (Liu and Yang 2007). Various strains of *Trichoderma* have been reported for their extracellular proteolytic profile and purified using chromatographic and isoelectric focusing methods (Kredics et al. 2005; Ridout et al. 1988). About 25% of the proteases have been contributed by *Aspergillus* alone among all fungal species (Sri Lakshmi et al. 2015).

9.5 Phylogenetic Relationship in Proteases of Fungal Origin

Phylogenetic tree determines the evolutionary history among organisms, genes, proteins, and whole genomes. This evolutionary tree can serve as the base for carrying interrelationships among genes and genomes by involving conserved and variable regions, regulatory sequences, and signature sequences (Choudhuri 2014). In this review, a comparative phylogenetic analysis of randomly chosen aspartic acid and serine-type proteases of thermophilic, mesophilic, and psychrophilic fungi based on their amino acid sequences retrieved from MEROPS database was depicted

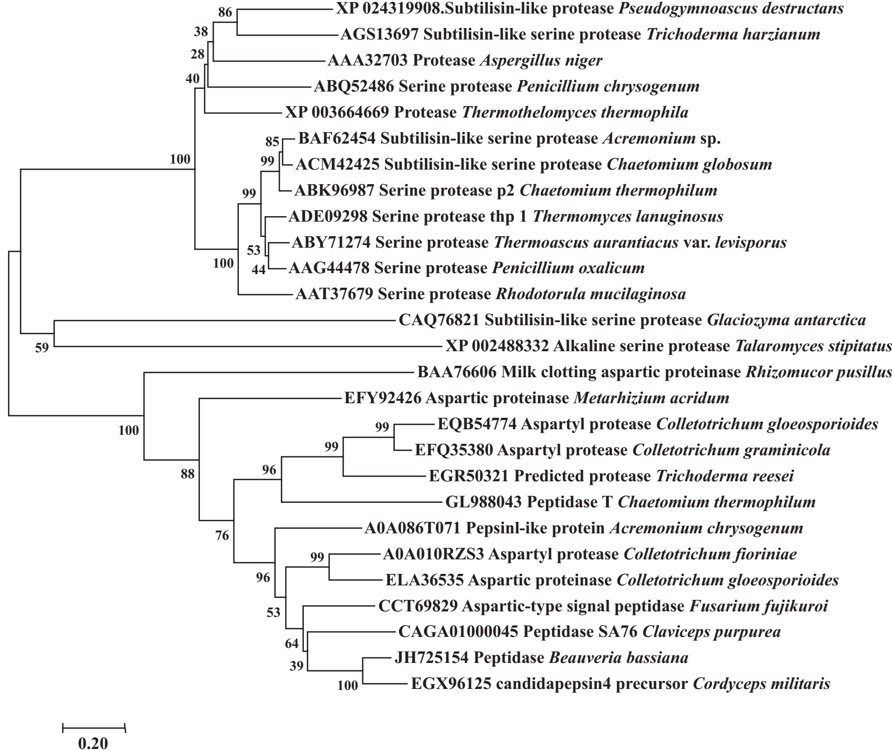

Fig. 9.3 Phylogenetic analysis of proteases from thermophilic, mesophilic, and psychrophilic fungi based on the amino acid sequences. The tree was prepared in MEGA version 7 based on neighbor joining method, and bootstrap values are given at nodes

and showed separate but close proximity and clustering of serine and aspartic acid proteases among thermophilic, psychrophilic, and mesophilic organisms (Fig. 9.3).

9.6 Properties of Fungal Proteases

Fungi are known to produce a variety of proteases including serine, aspartic, chymosin-like, chymotrypsin-like, and metalloproteases. The size of the proteases ranges from 16 to 97 kDa. These proteases are active over acidic 3–5.5 (Mandujano-González et al. 2013; Sun et al. 2018), neutral 6–7 (Hu et al. 2012; Aissaoui et al. 2017), and alkaline pH range 8–11 (Li and Li 2009; Niyonzima and More 2015) (Table 9.1) and temperature range from 30 to 65 °C (More et al. 2013; Hu et al. 2012). The fungal proteases are generally produced by submerged and solid-state fermentation and have suitability for use in industrial applications including detergent, cheese-making, dehairing, feather-degrading, meat processing, and antibacterial and pharmaceutical application like HIV-inhibitory activity (Table 9.1).

Table 9.1 Properties of proteases reported from different species of fungi

Organism	Protease	Molecular weight (kDa)	pH	Temperature (°C)	Possible applications	Reference
Aspergillus clavatus	Serine	32	8.5	50	Detergent	Hajji et al. (2007)
Aspergillus clavatus	Aspartic	30.4	5.5	50	Cheese, food	de Silva et al. (2011)
Aspergillus foetidus	Aspartic	50.6	5	55	Biotechnological	Souza et al. (2017)
Aspergillus tamarii	Serine	45	8.5	45	Dehairing	Anandan et al. (2007)
Aspergillus terreus	Serine	16	11	50	Detergent	Niyonzima and More (2015)
Aspergillus ustus	Serine	32	9	45	Detergent	Damare et al. (2006a)
Beauveria sp.	Subtilisin-like	29	9	50	Detergent	Shankar and Laxman (2015)
Chaetomium thermophilum	Serine	40.84	8	60	Industrial	Li and Li (2009)
Cunninghamella echinulata	Serine	33	4.5, 10	30, 60	Detergent, leather	More et al. (2013)
Cerrena albocinnamomea	Metallo	39.7	7	45	Fibrinogenolytic	Hamada et al. (2017)
Engyodontium album	Serine	28.6	11	60	Detergent	Chellappan et al. (2011)
Mucor subtilissimus	Chymotrypsin-like	97		37	Fibrinolytic	Nascimento et al. (2016)
Penicillium sp.	Serine	28	9	37	Collagenolytic	de Albuquerque et al. (2017)
Phanerochaete chrysosporium	Aspartic	38	4.5	50	Cheese production	da Silva et al. (2017)
Rhizomucor miehei	Aspartic	50.6	5.5	55	Food	Sun et al. (2018)
Scopulariopsis spp.	Serine	15	9	50	Detergent	Niyonzima and More (2014)
Sporisorium reilianum	Aspartyl	41	3	45	Biotechnological	Mandujano-González et al. (2013)
Termitomyces albuminosus	Serine	30	10.6	60	Detergent, leather	Zheng et al. (2011)
Termitomyces clypeatus	Chymosin-like	29	5	45	Cheese-making	Majumder et al. (2015)
Thermoascus aurantiacus	Serine	59.1	8	50	Industrial	Li et al. (2011)
Trametes cingulata	Serine	31	9	60	Detergent	Omrane Benmrad et al. (2016)
Trichoderma atroviride	Serine	21	8–9	50–60	Feather-degrading	Cao et al. (2008)
Trichoderma harzianum	Serine	20	7	40	Antibacterial	Aissaoui et al. (2017)
Xylaria hypoxylon	Aspartic	43	6–8	65	HIV-1 inhibitory	Hu et al. (2012)

9.7 Engineering of Proteases for Improved Performance

Various microbial strains have been modified by using gene editing tools including site-directed mutagenesis (SDM) and recombinant DNA technology for high yields of proteases (Fig. 9.4). The genes encoding proteases have been successfully isolated from fungal species, cloned in suitable vectors, and expressed in heterologous host for their efficient production using recombinant DNA technology. The genes encoding serine proteases have been characterized from *Aspergillus nidulans*, *Chaetomium thermophilum*, and *Thermoascus aurantiacus* and expressed in *Pichia pastoris* for improved production (Castro-Ochoa et al. 2013; Li et al. 2011; Li and Li 2009). Mostly, these proteases are secreted as inactive precursors which undergo autocatalytic processing after maturation. Such mechanisms have been reported in serine and aspartic acid proteases of *Mucor pusillus* and *Rhizopus niveus*. Aspartic acid proteases from *Aspergillus awamori*, *A. oryzae*, *A. saitoi*, and *Rhizopus niveus* were cloned and expressed in *E. coli* and yeast cells for efficient production (Rao et al. 1998).

Similarly, alkaline proteases from *Aspergillus oryzae* were first expressed in *S. cerevisiae* followed by *Zygosaccharomyces rouxii* for high yields (Ogawa et al. 1990). *Cephalosporium acremonium* have been reported in the cloning of extracellular proteases into *S. cerevisiae* (Isogai et al. 1991). The genes encoding serine proteases from *Chaetomium thermophilum*, *Thermoascus aurantiacus*, and *Tritirachium album* have been expressed in *Pichia pastoris* and *E. coli*, respectively (Samal et al. 1989; Li et al. 2011).

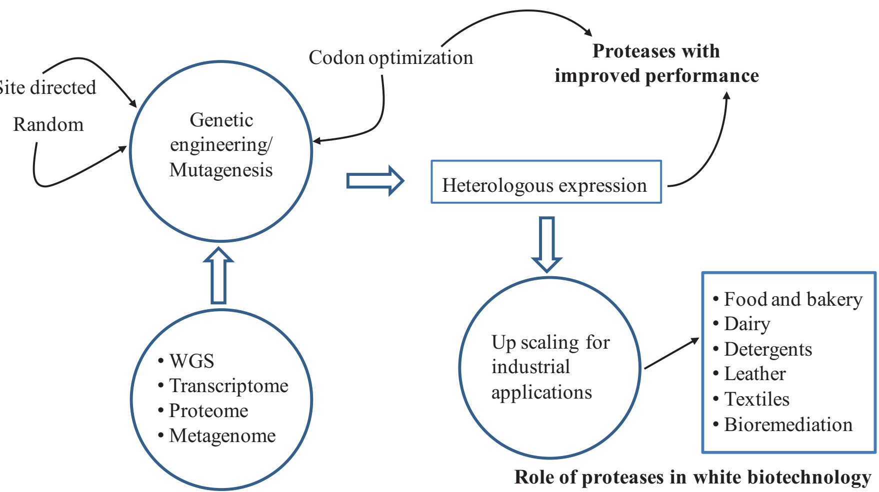

Fig. 9.4 Schematic illustration showing identification and mining of candidate genes of proteases, molecular tools for engineering of proteases for improved performance, and their utilization in industrial applications

Besides the production of engineered strains, molecular tools also help in making changes in the nucleotide sequences for producing proteins with altered function known as protein engineering. Various proteases have been engineered at the protein level for enhanced stability, substrate specificity, and improved catalytic efficiency at high temperature. The structure-function relationship of genes encoding subtilisin-like serine proteases has been resolved which shows variation in protein size at nucleotide level due to autocatalytic processing (Salwan et al. 2018). *Rhizopus niveus* produce pepsin which has been engineered for its substrate specificity by involving Asp77 and lysine in the P-1 position (Rao et al. 1998). Similarly, Asp76 has been replaced with serine residue in order to enhance the substrate specificity of aspergillopepsin I to basic substrates by using site-directed mutagenesis. Further, site-directed mutagenesis has been successfully employed to enhance thermostability in cysteine-free enzymes by incorporating incorporate disulfide bond. Ikegaya et al. (1992) have reported engineering of Alp protease from *Aspergillus oryzae* for enhanced thermostability and thermoresistance by the introduction of two disulfide bonds by replacing residues Ser69, Gly101, Gly169, and Val200 with cysteine. Similarly, oxidant and solvent stability in alkaline proteases has been introduced by replacing oxidation-sensitive amino acids with oxidation-resistant amino acids using SDM.

Further, improvement and modification of enzymes is being accomplished by using directed evolution (DE) which involves DNA shuffling, random priming recombination, and staggered extension process (StEP) approach (Jaeger et al. 2001). Various commercialized subtilisins have been engineered for solvent stability, thermal stability, and high substrate specificity using DE approach. This technique does not require prior knowledge on structure, function, and catalytic behavior of enzymes.

9.8 Proteases in White Biotechnology

Biotechnological applications in industrial sector are characterized by color like red for pharmaceutical, green for agricultural, and white for industrial biotechnology. The term white biotechnology involves the production of biological products produced from living cells and their enzymes (Chambergo and Valencia 2016). White biotechnology involves the production of food products, brewing and baking, detergents, textile, leather, paper and pulp, and bioremediation (Fig. 9.4). With the advances in molecular tools, metabolic engineering and enzyme technology with cost-effective benefits and environmental concerns have made a significant impact on the use of white biotechnology than before. Fungi are well-known species for the production of biotechnological products such as enzymes which are secreted in large amount outside the cells. Various species of fungi including *Aspergillus niger*, *A. melleus*, *A. oryzae*, *Fusarium* spp., *Penicillium* spp., *Rhizopus oryzae*, *Trichoderma harzianum*, and *T. reesei* have been reported for the production of homologous or heterologous proteins with ease of downstream processing like purification, recovery,

and storage. Among all enzymes, proteases are promising candidates because of their demand in biotechnological applications and for the development of eco-friendly technologies (Saxena et al. 2014, 2015; Trincone 2013).

9.8.1 Role of Proteases in Food and Dairy Industry

Fungal proteases have an important role in food industries for preparing nutritional protein hydrolysates, food formulations of newborns, dietary supplements, and processing of fruits for juices and soft drinks (Neklyudov et al. 2000). *Schizophyllum commune* has been reported for its suitability as cleaning-in-place (CIP) in the dairy industry. Proteases have a role in hydrolyzing casein during cheese production. Fungi like *A. niger* var. *awamori*, *Endothia parasitica*, *Mucor miehei*, *Penicillium* spp., and *Rhizopus oryzae* are reported and approved by FDA for cheese production (Yao et al. 2009; Adrio and Demain 2014; Kumar et al. 2017).

9.8.2 Role of Proteases in Brewing and Baking

Proteases are also used in baking industry like gluten for biscuits preparation and prevent sticking of commercial pastries from aluminum utensils. Protease like prolyl endopeptidase obtained from *Aspergillus niger* has been reported for the degradation of gluten peptides which can withstand the acidic environment of the stomach (Heredia-Sandoval et al. 2016). Fungal proteases from *Aspergillus oryzae* and *A. niger* have been reported for the production of sourdough breads, biscuits, cakes, and other sweet baked products (Heredia-Sandoval et al. 2016).

9.8.3 Role of Proteases in Detergents

Proteases find application for their use as additive in laundry because they can catalyze hydrolysis of proteinaceous materials such as blood spots from the fabrics (Adrio and Demain 2014). Different species of fungi including *Aspergillus clavatus*, *Beauveria* sp., *Engyodontium album*, and *Termitomyces albuminosus* have been reported for their suitability as additives in detergents.

9.8.4 Role of Proteases in Leather

Proteases are also involved in dehairing of animal skin which offers benefits over chemical treatment in soaking, dehairing, and bating for leather processing. Alkaline proteases display features such as time-saving, improved quality of leather and also

overcome pollution problems (Zambare et al. 2011). Proteases from *Aspergillus flavus* and *Conidiobolus coronatus* have been utilized in tanning during industrial processing of leather (Laxman et al. 2005; Malathi and Chakraborty 1991; Souza et al. 2015).

9.8.5 Role of Proteases in Textiles

Fungal proteases are also in large demand in textile industry for providing classical texture by removing gum and impurities on silk. The application of proteases in silk industry has led to the double gross returns to Indian sericulture (Gomaa 2013) because enzymatic treatment provides mechanical strength to the silk fiber and reduces environmental pollution (Sri Lakshmi et al. 2015).

9.8.6 Role of Proteases in Environmental Bioremediation

Fungi play an important role in environmental bioremediation by degrading pollutants such as textile dyes, effluents released from pulp and paper industry, leather tanning, petroleum hydrocarbons, and pesticides. Different species of fungi including *Aspergillus niger* has been reported for degradation of polychlorinated biphenyls; *Trichoderma* sp. for textile dye decolorization; *Candida* sp., *Mucor* sp., *Penicillium* sp., and *Rhizopus* sp. for petroleum products; *Aspergillus* spp. for degrading effluents from leather industry; and *Acremonium*, *Curvularia*, and *Pythium* for heavy metal tolerance (Deshmukh et al. 2016).

9.9 Conclusion and Future Prospects

Fungi are considered as potential candidates for industrial processes because they are rich source of producing extracellular proteases which find applications in detergents, leather, food processing and tenderization of meat, baking and brewing, and bioremediation. The wide substrate specificity of fungi and their metabolic potential offer advantages for producing proteases and their use in the area of white biotechnology. The use of microbial proteases in industrial processes offers reduced pollution concerns, improved economics, and sustainable production of biological products over chemical treatment (Gavrilescu and Chisti 2005). The native proteases from fungal species have disadvantages like reduced stability and less catalytic performance over wide temperature and pH range. To meet industrial demand, proteases have been engineered using recombinant DNA technology for heterologous expression, site-directed mutagenesis, and directed evolution of proteases with improved properties and catalytic efficiency. Only 5% of the fungal species have

been characterized which encourages further research on biotechnological aspects of fungi to explore their industrial potential.

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Chapter 10
Proteases from Endophytic Fungi with Potential Industrial Applications

Suchandra Mandal and Debdulal Banerjee

Abstract Proteases are enzymes that hydrolyse proteins and polypeptides into smaller constituents. Holding two-thirds of the global enzyme market, proteases are used to execute unique functions in industries of food, textile, detergent, therapeutics and environmental remediation. Fungi have emerged as the most dominant source of proteases due to several cultivational advantages over other sources. Expeditious industrial growth and emerging environment problems necessitate search for novel enzymes from more efficient fungal sources. Endophytic fungi form an uncharted ecological group of fungi with assorted synthesizing potential. Their efficacy has been proven within a short amount of time, as distinguished bioactive secondary metabolites have already been obtained from them. Commercial hydrolases are customarily isolated from soil-borne genera of fungi, but their endophytic counterparts offer a potential alternative, owing to recent findings that endophytes too display the same array of enzymes as do the soil fungi. Prospecting of endophytic fungi for protease production is not only promising, but there also exists the possibility of isolating and characterizing novel proteases that might be suitable alternatives for specialized industries. Several researchers have endorsed this postulate as they have discovered endophytic fungi with optimum protease producing capabilities and novel proteases with projected industrial applications prima facie.

10.1 Introduction

Proteases are enzymes that catalyse the cleavage of peptide bonds of proteins and polypeptides resulting in the formation of oligopeptides or free amino acids. They are also known as proteinases and polypeptidases. Proteases are ubiquitously present in all biological systems for carrying out proteolysis—a process vital for all forms of life. Proteolysis is necessary for both structural and functional aspects of a

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living being. Proteases are produced by every living cell either for intracellular operations or are secreted to the surroundings for nutritional and defensive functions. Besides being physiologically vital, they have also found over the years several critical roles to play in industries pertaining to health, food, textile and medicine (Correa et al. 2014). Their significance on the industrial level can be estimated by the fact that they claim up to 60% of the global enzyme market and constitute one of three major groups of industrial enzymes (Saran et al. 2007; Ningthoujam et al. 2009).

The industrial processes involving catalysis of proteins can be executed either enzymatically or chemically. Chemical degradation of proteins often leads to hydrolysates with undesirably modified amino acids and uncontrollable reactions. Chemical modification has been preferably substituted with highly specific biocatalysis. Proteases represent a reusable, sustainable environmental friendly alternative. Products formed from proteases are gaining popularity and preference among commoners as people are inclining towards natural products rather than chemically synthesized ones (Sumantha et al. 2006; Tavano 2013; Saxena et al. 2014, 2015; Suman et al. 2016; Verma et al. 2017).

Earlier, proteases were classified on the basis of some practical and functional facets, such as their catalytic actions (endoprotease and exoprotease), source (animal, plant or microbial), pH optima (alkaline, neutral or acidic), substrate specificity, etc. A more rational system was contrived by the Enzyme Commission (EC) under which all enzymes had been grouped into six classes. Proteases fall under class three, which comprises of hydrolases, and subgroup four—which characterizes enzymes with the ability to hydrolyse peptide bonds (EC 3.4). This class is further divided into families; six such families have been recognized hitherto—serine carboxy proteases (EC 3.4.16), metallo carboxy proteases (EC 3.4.17), serine proteases (EC 3.4.21), cysteine proteases (EC 3.4.22), aspartic proteases (EC 3.4.23) and metalloproteases (EC 3.4.24) (Whitaker 1994).

Classification of proteases on the basis of their functional pH range is one of the most feasible and workable basis as it can readily identify the type of industrial sector the protease can be applied to. Acidic proteases function optimally in the range of pH 0–6.0. They act on the breakdown of bonds involving aromatic amino acids bulky side chains at both sides of the cleaving bond. Acid proteases find use mainly in food industries. Neutral proteases are active in the pH range of 5.0–8.0. They have a characteristic high affinity for hydrophobic amino acids in the polypeptide chain and have low degree of hydrolysis. Their low thermal tolerance provides a mechanism of reaction control and facilitates achieving hydrolysates with limited hydrolysis. The proteases that show maximum activity in the neutral-alkaline (7.0–14) pH range are called alkaline proteases. They either have a serine centre or are of metallo-type. They are perhaps the most extensively studied among the three groups of proteases. This is due to their potentially huge marketability as they are useful in a variety of industries like detergent, food, pharmaceutical and leather industries (Sharma et al. 2017; dos Santos Aguilar and Sato 2018).

10.2 Applications of Enzymes Proteases in Industries

Proteases have served in the industries since time immemorial, the earliest application of these being in the food industry. The reaction involving degradation of proteins into smaller constituents has found disparate uses in various fields. Proteases have emerged as a significant enzyme group holding two-thirds of the global enzyme market. In addition to their conventional uses in dairy, bakery, leather and detergent industry, modern times are seeing proteases being exploited for some unconventional purposes, ranging from bioremediation to treatment of diabetes, cancer and AIDS (Ladenburger et al. 1997; Rao et al. 1998; Abdennabi et al. 2017; Yadav et al. 2016, 2017a, b). Their utility in a specific industry is generally a reflection of their chemical nature and optimum working conditions (Fig. 10.1). Some commercial applications of proteases are described below.

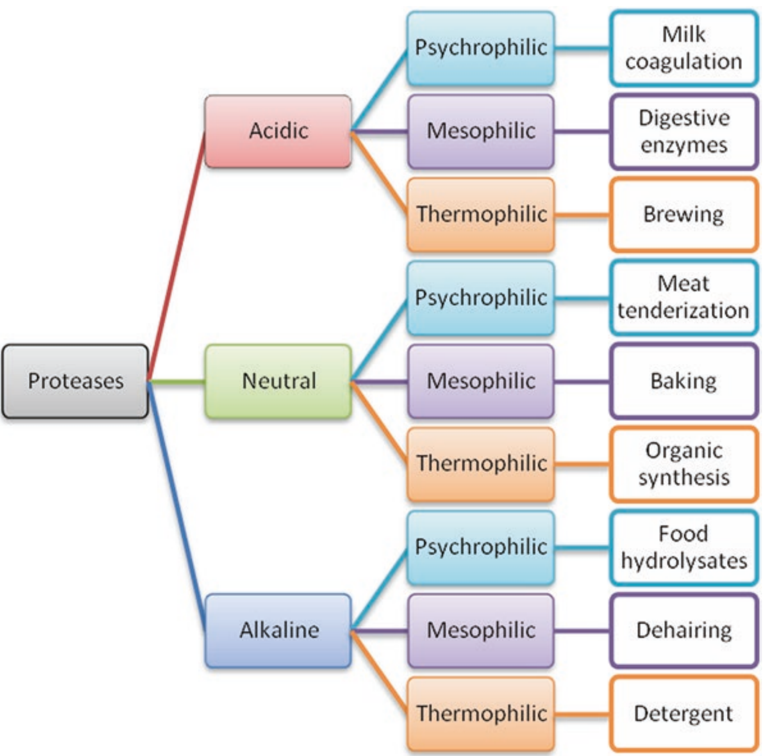


Fig. 10.1 Various functions served by proteases in the industries based on their types

10.2.1 Therapeutics

Proteases are digestive enzymes that are required for breakdown of proteinaceous food, providing essential amino acids to the body. Many pathophysiological conditions can cause obstruction in this normal catalytic process. Administration of protease formulations to such patients improves the digestion process (Craik et al. 2011; Rajput et al. 2016). Zenpep® (Eurand) is one such protease preparation available in the market for treating malabsorption of nitrogen in patients with cystic fibrosis. Patients of cystic fibrosis suffer from a decreased production and release of pancreatic enzymes which compromises their ability to absorb nitrogen from food sources. Zenpep® is a porcine-derived preparation of proteolytic and lipolytic enzymes that have tremendously helped people as a digestive aid. However, such replacement enzymes of porcine origin have caused allergies in humans. Trizytek™ (Eli Lilly) is another digestive aid currently in development that has shown promising results. The formulation comprises a lipase, amylase and an alkaline elastolytic protease from the fungus *Aspergillus melleus*. The oral delivery of the preparation has been shown to improve protein digestion in cancer and cystic fibrosis patients with pancreatic insufficiency (Littlewood et al. 2006; Wooldridge et al. 2009).

Cosmetic reconstruction and wound healing are medical techniques where proteases have been employed extensively. Proteases have been used in skin ointments for removal of necrotic tissue in skin ulcers, debridement of wounds, removal of keratin in acne or psoriasis, degradation of keratinized skin and elimination of human callus (Gupta et al. 2002a; Shubha and Srinivas 2017). Keratinolytic proteases are a potential resource for accelerating healing process by scar removal and renewal of epithelia. Cosmetic preparations of plant proteases papain and bromelain have been in use for regenerating skin through peeling and smoothing. They act by eliminating collagenous and keratinous debris, thereby removing dead cells from the epithelia and renewing the same. Proteases have been an additive in vaccine therapies for dermatophytosis. Collagenolytic proteases have been employed in treatment of sciatica in herniated intervertebral discs, in treatment of retained placenta and as a pretreatment for enhancing adenovirus-mediated cancer gene therapy. Penzyme is a commercial concoction of trypsin and chymotrypsin that can be helpful in treatment of psoriasis by digesting outer damaged layers of skin (Sim et al. 2000; Vignardet et al. 2001; Brandelli et al. 2010).

Antibody fragments are gaining importance in diagnostics and drug designing since the past few years. Generation of antibody fragments involves proteolysis of entire immunoglobulin molecules. Specific digestion of IgG molecules by papain, pepsin and ficin has been traditionally done to dissect the antibody molecule into fragments. Fragments of monoclonal antibodies provide advantage over whole antibody molecules due to their small size, faster diffusion and lower immunogenicity while maintaining specificity. These properties have led to their application in diagnostics, therapeutics and biopharmaceutical research (Holliger and Hudson 2005; Mótyán et al. 2013).

Proteases execute diverse functions ranging from the cellular to the organism level. They are involved in regulation of cascades of haemostasis and inflammation in the vertebrate system. Apart from being a crucial element in the normal physiology of cells, proteases also play an important regulatory part in pathophysiological conditions of an organism. The necessary role they have in completion of life cycle of pathogens and anomalous cells has led to the development of new drugs that target them for treating terminal diseases such as cancer and AIDS. Microbial proteases are used as immunostimulatory agents and in combinatorial treatment with antibiotics (Okumura et al. 1997). Studies done by Ladenburger and his team (Ladenburger et al. 1997) show that protease administration successfully delayed the onset of insulin-dependent diabetes mellitus in mice with autoimmune diabetes.

10.2.2 Food

The most notable purpose served by proteases has been in the food industry, particularly in cheese-making and bakery. The biochemical process of proteolysis is accountable for the modification of milk to cheese, and it has been traditionally performed using animal rennet (chymosin). Chymosin is the most suitable and preferable enzyme for cheese-making since it has high specificity for casein and its low thermal tolerance ensures that the enzymatic activity ceases upon cooking, which can otherwise cause bittering of cheese. But due to inadequate supply of this enzyme of animal origin, focus has shifted to microbial milk coagulants. An increasing number of cheese manufacturing industries have employed proteases of microorganisms that have been deemed GRAS (Generally Recognized as Safe) by the US Food and Drug Administration, like *Mucor miehei*, *Bacillus subtilis* and *Endothia parasitica*.

Proteases have also assisted in bakeries through their action of limited proteolysis on gluten. Gluten is an insoluble protein present in wheat, and it determines the viscoelastic and mass expansion properties and flavour of bakery dough. Pretreatment of dough using protease results in reduction of mixing time and improvement of loaf volume. Protease treatment is in general used to manipulate gluten strength to achieve multifarious bakery products each with unique flavour and properties. Endo- and exoproteases from *Aspergillus oryzae* (BakeZyme B500BG) and a neutral metalloprotease from *Bacillus amyloliquefaciens* (Neutrase®) have been utilized for degrading proteins in flour dough for preparing biscuits, cakes, crackers and cookies (Sawant and Nagendran 2014).

Proteases are important to the brewing industry too, as addition of protease can increase the growth of yeast in fermentation media, resulting in better and faster yield of alcohol. It also aids in extraction of nutritional proteins from malt and barley. Clarification of protein drinks, alcoholic beverages and fruit juices also requires protease. Proteases are also used in clarification of xanthan gum. Kojizyme and Flavourzyme are commercial fungal proteases that are used in fermentation of soy

sauce and seasoning and limited hydrolysis of proteinaceous food materials like meat, fish, casein, gelatin, etc. The protein hydrolysates formed from partial hydrolysis are valued as nutritional supplements (Ward et al. 2009).

10.2.3 Leather and Textile

Leather processing involves steps of soaking, dehairing, bating and tanning of the animal hide. Dehairing process could be carried out either chemically or enzymatically. Chemical processing utilizes strong alkali for soaking, followed by hydrogen sulphide application for dissolving hair roots. The extreme alkaline conditions and dangerous chemicals like hydrogen sulphide render the chemical method extremely hazardous for the workers. Moreover, the huge amount of chemical wastewater contributes significantly to environmental pollution. Processing by proteases is a much safer option both for industrial workmen as well as the environment. Protease treatment reduces soaking time by accelerating water absorption. This reduces the requirement of water and minimizes effluent release. Protease operates by removing non-collagenous materials of leather and dissolving non-fibrillar proteins like globulins and albumins. Physical conditions of leather processing happen to be optimal for alkaline proteases. Application of alkaline proteases with sodium chloride and hydrated lime is efficient in dehairing animal skin. Various combinations of proteases from *Bacillus* and *Aspergillus* along with trypsins have been used in leather processing. Enzyme use has been shown to improve leather texture and quality (Ward et al. 2009; dos Santos Aguilar and Sato 2018).

Proteases are a valuable resource for the silk industry. Raw silk consists of two protein components – sericin (22%) and fibroin (76%). Fibroin is the major component that forms the finished product. Sericin is a water-soluble protein that forms a protective and adhesive layer over the fibroin. Removal of sericin, known as degumming or silk scouring, is necessary to achieve strength, texture, lustre and colour in the finished fabric. It is accomplished through digestion of raw silk by proteases. This method is superior to soap treatment as protease action can be controlled to get desired strength of silk fabric and it is environmentally friendly too (Gulrajani and Gupta 1996).

10.2.4 Detergents

The idea of incorporating proteases to detergent was pioneered by German chemist Otto Röhm. He obtained a patent in 1913 for using tryptic enzymes of animal origin with laundry detergent and formulated the first enzyme detergent named Burnus® with his associate Otto Haas. Unfortunately, the formulation did not gain popularity due to inefficiency. This was later attributed to the inactivation of the tryptic enzymes in the alkaline conditions produced by the detergent. The first preparation of

detergents with proteolytic enzymes that gained popularity and acceptance among the common mass was introduced by Novo Industri in the year 1961. It comprised of an alkaline protease from *Bacillus licheniformis* that was stable at high pH range of 8–10. Since then, proteases have become the most sought-after enzymes in the detergent industry. Mostly serine alkaline proteases are used for this purpose. Addition of protease provides several perks such as easy removal of proteinaceous dirt (blood, body secretions, milk, fish and meat stains, etc.) and reduction in amount of water required for washing, minimizing the physical effort and time that go into doing laundry. Enzymatic detergents have made cold washing more effective, thus saving energy. Various proteases capable of functioning in a variety of pH and temperature ranges are added to detergents, and this has given way to a new class of detergents that have minimum impact on the environment (Samal et al. 1990; Ward et al. 2009; Valls et al. 2011; Sahay et al. 2017; Saxena et al. 2016; Suman et al. 2015).

10.2.5 Organic Synthesis

Radically, proteases have been used to disintegrate polypeptides. Proteolysis involves an equilibrium reaction between synthesis and disintegration, the equilibrium being driven and controlled by the amount of water in the reaction solution. By manipulating the moisture content and through use of appropriate solvent, the reverse reaction can be coerced. This is termed Protease-Mediated Peptide Synthesis (PMPS). A remarkable use of protease in organic synthesis is the industrial manufacture of aspartame, an artificial sweetener used as a sugar substitute. A heat-stable extracellular Zn^{2+} metalloprotease from *Bacillus thermoproteolyticus* is employed for reverse hydrolysis that yields the dipeptide aspartame (Kühn et al. 2002; Ward et al. 2009; Birrane et al. 2014).

10.2.6 Research

Many techniques in biological research avail proteases. Proteinase K is a well-known protease that is exploited in laboratory-scale biochemical processes. It was first described by Ebeling and his team in 1974 from the fungus *Tritirachium album*. It was found to possess a superlative proteolytic, specifically keratin-hydrolysing, property. It is applied in nucleic acid isolation procedure for removing unwanted protein components of the cells and also to inactivate nucleases that might attack the nucleic acids, thereby increasing extent of purification and yield of the isolated DNA/RNA (Ebeling et al. 1974; Mótyán et al. 2013).

Tissue culture techniques also require protease application for dissociation of intact tissue and liberation and isolation of detached viable cells. Separation of cells involves digestion of junctions connecting the cells and dissolution of the

extracellular matrix. Proteases form an important component of the array of enzymes utilized in this process. During flask culture of tissues also proteases are used to separate the cell layer from the surface of flask by dissolving the protein bridges. Trypsin is generally used for this purpose (Canavan et al. 2005; Huang et al. 2012).

Proteases are nowadays proving to be tremendously helpful in management of industrial and household wastes, for accelerating the process of degrading waste material, in wastewater management and other bioremediation processes. Proteases are used in cleaning solutions for contact lenses for removing proteinaceous debris. They also find use in photography and biomedical industries for dissolving gelatin off scrap films of photos and X-rays that allow recovery of silver from the films. Proteases perform dynamic functions, and interests are ever growing in finding proteases with unique and novel biological properties (Anwar and Saleemuddin 1998; Nielsen and Oxenboll 1998; Kumar and Takagi 1999; Gupta et al. 2002b; Harrison and Bonning 2010; Hasan et al. 2013; Alberto et al. 2016; Rajput et al. 2016). A synopsis of commercial proteases currently in use, with their respective sources of origin, is provided in Table 10.1.

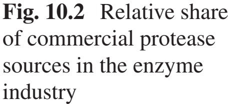
10.3 Proteases from Fungi: How They Are Prevalent and What Advantages They Have

A few decades ago, animals used to be the sole source of industrial enzymes. Pigs and cows were slaughtered, and proteases recovered from them were utilized in the industries. A few proteases were described from plants later on, and they served some particular functions in commercial sectors. Remarkable expansion of the enzyme trade occurred after realization that microbial sources surpass animal and plant sources in terms of enzyme production. Microbial production of enzymes is more profitable due to several reasons. Microorganisms (bacteria and fungi) have limited space requirement (Najafi et al. 2005). The enzymes they produce are extracellular in nature, i.e. they are secreted into the culture media, and this makes the downstream processing easier. They can grow in simple media composition with a rapid growth rate and faster rate of enzyme production. They are also amenable to genetic manipulation and recombination (Anbu et al. 2013). The proteases obtained from microbes also have an edge over animal and plant proteases as they have been found to have greater thermal stability, allowing longer shelf life (Maria et al. 2005; Sharma et al. 2014). They have minimum loss of function even over adverse storage conditions. Microorganisms provide a reliable and consistent yield with constant composition along predictable and controllable fermentation conditions.

Over the past few years, fungi have outperformed other enzyme sources, and currently about 60% of industrial enzymes are of fungal origin (Fig. 10.2). Fungi have become more acceptable as industrial enzyme synthesizers than their bacterial counterparts due to greater efficiency of the fungal mycelia, easy separation of mycelia from the culture media and greater biochemical diversity of proteases found

Table 10.1 Commercial proteases and their sources

Protease	Source	Class	Optimum pH	Molecular mass (kDa)
Bacterial origin				
Endoproteinase Asp-N	*Pseudomonas fragi*	Metalloprotease	6.0–8.0	27
Endoproteinase Glu-C (V8 protease)	*Staphylococcus aureus*	Serine protease	8	27
Endoproteinase Lys-C	*Lysobacter enzymogenes*	Serine protease	8	30
Thermolysin	*Bacillus thermoproteolyticus*	Metalloprotease	7.0–9.0	37.5
Subtilisin	*Bacillus subtilis*	Serine protease	7.0–11.0	30
Clostripain (endoproteinase-Arg-C)	*Clostridium histolyticum*	Cysteine protease	7.1–7.6	59
Fungal origin				
Proteinase K	*Tritirachium album*	Serine protease	7.5–12.0	18.5
Carboxypeptidase P	*Penicillium janthinellum*	Serine protease	4.0–5.0	51
Carboxypeptidase Y	*Saccharomyces cerevisiae*	Serine protease	5.5–6.5	61
Flavourzyme	*Aspergillus oryzae*	Serine Carboxyprotease	5	67
Plant origin				
Papain	*Carica papaya*	Cysteine protease	6.0–7.0	23
Ficin	*Ficus septica*	Cysteine protease	6.0–8.0	23.8
Bromelain	*Ananas sativus*	Cysteine protease	4.5–7.5	33.2
Actinidain	*Actinidia chinensis*	Cysteine protease	6.0–6.5	27
Animals origin				
Chymotrypsin	Bovine	Serine protease	7.5–8.5	25
Trypsin	Bovine	Serine protease	8.0–9.0	23.5
Endoproteinase Arg-C	Mouse submaxillary gland	Serine protease	8.0–8.5	30
Pepsin	Porcine	Aspartic protease	2.0–4.0	34.5
Elastase	Porcine	Serine protease	7.8–8.5	25.9
Exopeptidase Carboxypeptidase A	Bovine	Metalloprotease	7.0–8.0	34.5
Carboxypeptidase B	Porcine	Metalloprotease	7.0–9.0	34.6
Cathepsin C	Bovine, Turkey	Cysteine protease	5.5	210
Acylamino-acid-releasing enzyme	Porcine	Serine protease	7.5	360
Pyroglutamate aminopeptidase	Bovine	Cysteine protease	7.0–9.0	70–80



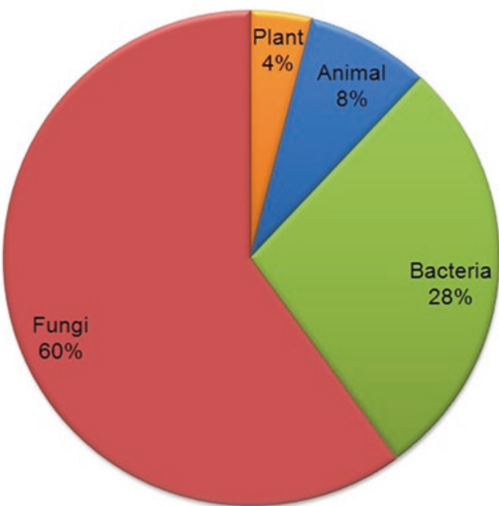


Fig. 10.2 Relative share of commercial protease sources in the enzyme industry

in fungi (Ningthoujam et al. 2009; Sharma et al. 2017). Serine, threonine, metallo-, aspartic and cysteine protease and other uncharacterized proteases have been produced from fungi, and they have been employed in manufacturing food, beverages, leather and textile and in simplifying the processing of raw materials (Rawlings et al. 2004; Maria et al. 2005). The excellent enzyme-synthesizing abilities of fungi are accredited to their saprotrophic and parasitic mode of nutrition. This lifestyle demands exhibit of a gamut of degrading enzymes that dissolve host tissues or disintegrate dead tissues so that the released nutrients can be absorbed by the fungi. Extracellular proteases released by fungi hydrolyse peptides and facilitate nitrogen uptake (Schulz et al. 2002; Suryanarayanan et al. 2012).

10.4 Need to Look for New Sources

Insatiable demand for industrial enzymes has led to extensive research efforts into optimizing and maximizing production from existing protease sources (Kirk et al. 2002). Industrial growth has caught up a breakneck speed, and to meet demands, research is ever-going for finding novel and more proficient protease sources as well as organisms that produce unprecedented kinds of proteolytic enzymes. Novel proteases are providing solutions to many previously unresolved challenges in the biomedical and biotechnological sectors. When it comes to enhancement of enzyme synthesis, natural selection has always been preferable to combinatorial chemistry (Schulz et al. 2002).

Increasing health consciousness of the society has led to rejection of many synthetic products. Demand for products from natural sources has increased, and focus has shifted to screening microorganisms for finding bioactive compounds (Strobel

and Daisy 2003). Although fungi have been the dominant enzyme producers in industries, it is perplexing that only a handful of fungal species are exploited on the industrial scale. Species of *Aspergillus* (particularly *A. niger* and *A. oryzae*), *Humicola*, *Penicillium*, *Rhizopus* and *Trichoderma* have been prepotent in commercial bioproduct synthesis (Østergaard and Olsen 2010; Correa et al. 2014). It is safe to presume that out of an estimated 1.5 million members, many undocumented and uninvestigated fungal species are promising and potent sources of bioactive products (Hawksworth 1991; Peterson et al. 2011).

Bioactive product discovery relies heavily on the strength of culture collection for screening and requires exploration of atypical environments (Hyde 2001). It is a general notion that organisms from unusual habitats possess unusual characters corresponding to the habit requirements. The terrestrial environment has been thoroughly scanned, and resources in this front have been exhausted, so there is an urgent need to scrutinize other ecological groups of fungi (Correa et al. 2014). Studies on endophytic fungal flora have been initiated recently after it was comprehended that plant tissues are not sterile but are indeed inhabited by a large number of fungi and bacteria. Endophytic fungi have been explored in terms of their species diversity and bioactive metabolite production. Within a short amount of time and with a handful of studies, endophytes have proved to be an unexplored repository of natural products and have already offered distinguished bioactive metabolites. Fascinatingly, most studies on screening endophytic fungi are for secondary metabolites. Prospecting of endophytic fungi for protease production is not only promising, but there also exists the possibility of isolating and characterizing novel proteases that might be suitable alternatives for specialized industries (Alberto et al. 2016).

10.5 Endophytic Fungi as Enzyme Synthesizers

10.5.1 What Are Endophytic Fungi?

Endophytic fungi are a highly diverse ecological group of fungi circumscribed by their habitat- internal tissues of plants. They live inside plant tissues without making their presence apparent. They are a polyphyletic group of taxonomically and metabolically diverse fungi that make up a significant component of microbial diversity in the environment (Wilson et al. 1991; Arnold 2007; Rana et al. 2016a, b, 2017; Suman et al. 2016). In the initial years following their discovery, endophytes were thought to have a neutral relation with their host plant. But later investigations, beginning from 1970, revealed that they in fact form a mutual partnership with their host. This was corroborated by the finding that clavicipitaceous members residing inter- and intracellularly in grasses deter herbivore feeding. They compensate for the inability of grasses to produce toxic secondary metabolites. The repugnant toxic alkaloids produced by the endophytes thwart consumption of the plant

by herbivores. Subsequent studies established that endophytic fungi aid in survival and health of their hosts through production of heterogeneous bioactive metabolites. The relation is mutualistic in the sense that the fungi help the plant through biotic and abiotic stresses and, in turn, derive nutritional carbon source from the host. Consequently, they are predominantly found in the sink tissues of plants, regions of sucrose unloading such as leaf sheaths and pith (Hinton and Bacon 1985). They are transmitted either vertically, through seeds or vegetative propagation of host plant, or horizontally, invading the plant tissues via natural (stomata, lenticels) or artificial (mechanical injury) openings (Carroll 1988). Their invasion and ramification inside the host tissues require display of a battery of degrading enzymes, and survival inside the host involves active production of primary and secondary metabolites.

10.5.2 Their Synthesizing Abilities

Endophytes exist in symbiotic partnership with their hosts. They actively synthesize diverse compounds in their habitat that facilitate better nutrient uptake by their host, enhancing host health and fitness. They prevent pathogen invasion and upregulate plant defense system. In the ecological niche they occupy, they constantly engage in biological warfare with other microbial species, many of which are phytopathogens, for space and nutritional requirements. This necessitates production of antagonistic substances that endow the endophytes with better chance of survival. Due to their ability to live inside plant tissues facing defense chemicals of the host plant, their capacity to detoxify and transform bioactive molecules can be rightly predicted to be employed on an industrial scale (Suryanarayanan et al. 2012). Endophytic fungi isolated from many angiosperms and gymnosperms have been bioprospected and found to be producing unique structures, including alkaloids, benzopyranones, chinones, flavonoids, phenols, phenolic acids, quinines, isocoumarin derivatives, steroids, peptides, terpenoids, tetralones and xanthones (Tan and Zou 2001; Strobel et al. 2004).

There have been many instances where endophyte of a particular plant was observed to produce phytochemicals specific to the plant. Tan and Zou (2001) postulated that millions of years of co-evolution has led to genetic recombination between such endophytes and their hosts, and fungi show a greater affinity towards accepting foreign genetic material through horizontal gene transfer. Genes for novel product synthesis might have been shared in this way between the two symbiotic partners through evolutionary time. Many of the secondary metabolites produced by endophytes have diverse applications in agrochemicals, medical therapy, as anti-parasites, immune-modulatory agents, antioxidants, cytotoxic agents, etc. Endophytic fungi have been found to produce such chemicals in independent cultures—a feature that renders them suitable as bioactive product sources at the industries. Several studies have substantiated their ability to produce distinctive substances that possess bioactivity, such as novel antibiotics; antimycotics;

immunosuppressants; anticancer, antiviral and volatile organic compounds including volatile antimicrobials; insecticides; and antidiabetic compounds (Strobel and Daisy 2003). Many fungi isolated from plants have been screened for enzyme synthesis potential, but majority remain unexplored, providing huge scope for finding alternative sources of enzymes (Alberto et al. 2016).

10.5.3 Their Promising Candidature as Protease Sources

Commercial hydrolases are customarily isolated from soilborne genera of fungi like *Aspergillus*, *Penicillium* and *Rhizopus* (Lee et al. 2014). But their endophytic counterparts offer a potential alternative as it has been established that endophytes too display the same array of enzymes as do the soil fungi (Promputtha et al. 2007). Enzyme synthesis is an integral part of endophytic life cycle as enzymes have several important functions:

- Hydrolytic enzymes dissolve the lignocellulosic material of plant cells to enable fungal mycelia to penetrate the host surface and ramify inside the host plant tissues.
- Enzymes disintegrate sources of nitrogen, phosphorus, calcium, etc. external to the plant roots and enable the plant to absorb these otherwise inaccessible sources.
- Enzymes are necessary for the absorptive mode of nutrition of fungi. Enzymes breakdown complex food materials, such as starch and sucrose obtained from the host tissues, into simpler units that get absorbed by the fungal mycelia.
- Enzymes prevent pathogen invasion and expansion by targeting substrates on the surface and interior of the pathogen's anatomy.

Role of enzymes extends after senescence of host tissue, as fungi's lifestyle shifts to saprotrophic mode. From that point onwards, the enzymes perform degradation of the dead organic matter, and the decaying tissues supply nourishment to the fungi (Maria et al. 2005). There have been a number of studies on enzymatic profiling of endophytic fungi. Endophytic fungal isolates have been scrutinized for general or specific hydrolytic enzymes such as amylase, protease, lipase, cellulase, tannase, laccase, etc. and have given promising results (Sunitha et al. 2013). Caldwell et al. (2000) reported that *Phialophora finlandia* and *P. fortinii*, endophytic fungi isolated from alpine plant communities, were able to breakdown complex forms of phosphorus, nitrogen and carbon found in plants. Choi et al. (2005) screened the endophytic fungi for their ability to produce lignocellulases, amylase, cellulase, ligninase, pectinase and xylanase. Maria et al. (2005) performed similar studies on fungi isolated from mangrove fern *Acrostichum aureum* L. and mangrove angiosperm *Acanthus ilicifolius* L. Screening tests have revealed that all endophytic fungal strains do not share the property of enzyme synthesis, and this specialization arises due to their specific adaptation to the environment in which their host plants are found (Sunitha et al. 2013).

Adequate literature is present in bioprospection of fungi for secondary metabolite production, but it is scarce in case of enzyme profiling of endophytic fungi. Their huge potential and promising results in preliminary tests rationalize their candidature as potent enzyme producers. Sunitha et al. (2013) postulated that the possibility of endophytic fungi actually being weak parasites or latent pathogens warrants their protease producing capacity. Since nitrogen is an important macronutrient that endophytes derive from plants, protease can be found as an integral and crucial component of the assemblage of enzymes made by endophytes. There is additional likelihood of endophytes acquiring novel protease genes from its host plants over evolutionary time (Priest 1984; Vasundhara et al. 2016). Pavithra et al. (2012) conjecture that since extracts of Basil (*Ocimum* sp.) containing proteases are effective in control of diabetes, protease enzymes from the endophytic fungi residing in this plant will have similar properties. Table 10.2 depicts some proteases described from fungi of endophytic origin.

10.5.4 Advantages They Might Have Over Present Fungal Sources

Fungal enzymes predominant in the industries are all from soil fungi. These enzymes are also produced by endophytic fungi, their distinguishing character being that they are biochemically adapted to the endophyte's natural environment (Borges et al. 2009). *Aspergillus niger*, a soil fungus regarded as GRAS, used widely for obtaining various enzymes, has been recently found as an endophyte of several plants (Ward et al. 2005; Meijer et al. 2011). The industries not only seek sources with better production but also novel proteases and newly discovered functions of existing proteases. Attaining novel structures from fungal cultures is always lucrative

Table 10.2 Endophytic fungi and their proteases

Endophyte name	Source	Type of protease	Molecular mass	Reference
Acremonium typhinum Morgan-Jones & Gams	*Poa ampla* Merr. cv service	Serine endoproteinase (proteinase At1)	34 kDa	Lindstrom and Belanger (1994)
Epichloë festucae	*Lolium perenne* cv. Nui	Subtilisin-like proteases (SLPs)	–	Bryant et al. (2009)
Pestalotiopsis microspora	*Psidium guajava*	Serine protease	21 kDa	Russell et al. (2011)
Xylaria psidii KT30	*Kappaphycus alvarezii*	Serine protease	71 kDa	Budiarto et al. (2015)
Umbelopsis isabellina	*Betula* sp. and *Abies* sp.	Aspartic protease	70 kDa	Mayerhofer et al. (2015)
Xylaria curta	*Catharanthus roseus*	Metalloprotease	33.76 kDa	Meshram et al. (2016)

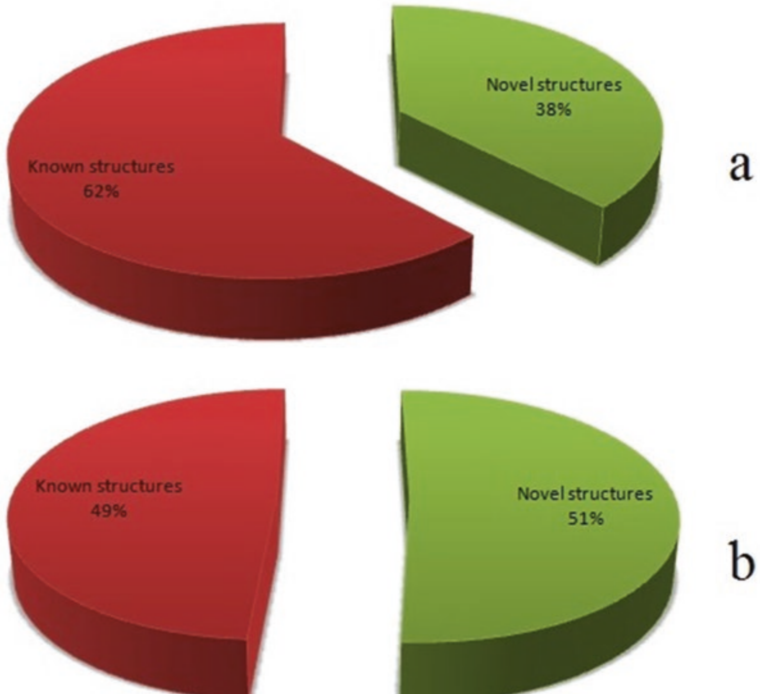


Fig. 10.3 Relative abundance of novel and known structures found in the culture broth of (**a**) soil fungi and (**b**) endophytic fungi

and sought after. A comparative study of structure determination between soil fungi and endophytic fungi to determine the percentage of novel structures in their metabolome revealed that endophytic fungi have a higher proportion of unknown structures (51%) in their culture extracts than soil fungi (38%) (Fig. 10.3).

Since isolation, characterization and structure determination of a new compound is a tedious process, it is intelligent to screen endophytic fungi, for they offer better probability of finding novel products (Schulz et al. 2002). Zaferanloo and her team (Zaferanloo et al. 2013) showed through their work how relatively easy it is to rapidly screen endophytic fungi using low-cost substrates and identify industry-ready isolates with excellent synthesizing capacity. Enzyme biosynthesis pathways and products in endophytic fungi are adapted to a particular ecological condition and attuned to perform discrete functions. Such enzymes are often more stable and harmless than those obtained from other sources (Raju et al. 2015). Production of enzymes by endophytic fungi is more eco-friendly and sustainable and provides better quality control (Tenguria et al. 2011). Li and her associates (Li et al. 2012a) suggest through their work that endophytes isolated from plants of Baima Snow Mountain are adapted to cold climate and their enzymes might be cold attuned. Isolation of those enzymes can be helpful in biotransformation of heat-labile

substances. Enzymatic profiling proves helpful in screening capable fungi, and further characterization can identify proteases that can cater to various industrial demands (Sunitha et al. 2013; Alberto et al. 2016).

10.6 Screening of Endophytic Fungi for Protease Production

10.6.1 Isolation from Appropriate Niches

The foremost step in searching potent protease-producing endophytic fungi is the selection of host plant. Two aspects have to be kept under consideration while choosing a plant specimen- ecological habitat of the plant and its established and potential phytochemistry. Among all geographical regions of the world, the tropical rainforests are speculated to harbour more than 60% of the world's biodiversity. Plants of this region have inevitably a richer diversity of endophytic fungi than plants of other terrains. They have a larger number of representatives that are potential candidates for biosynthesis. Endophytic fungi from other phytogeographic regions might be less diverse, but there could be some unique strains that one might not find in the hot and humid equatorial regions. Choice of the plant itself is also equally important and depends on the phytochemical profile of the plant. For this reason, plants with proven medicinal properties or ethnomedicinal applications are favoured for endophyte isolation (Zaferanloo et al. 2013). Here the concept of shared properties between host and endophyte provides rationale for selection. Environmental screening programmes are set up to evaluate and select appropriate samples from a region's vegetation. These programmes are beneficial for discovering novel enzyme synthesizers with distinct properties, and the ecological habitats of microorganisms help in anticipation of properties of the enzymes.

After selection and collection of plant sample, it must be quickly processed for isolating endophytes. Isolation is done using various culture media. Composition of the media is adjusted and modified to support growth of desired organism and suppress the growth of other undesirable organisms that might be present in the incubated tissue or could be contaminants coming from aseptic conditions. Different workers have used different isolation media, such as water-agar medium (WA), Sabouraud dextrose agar (SDA) media, malt extract agar, Czapek dox agar and potato dextrose agar (PDA) media (Patil et al. 2015; Meshram et al. 2016; Abdennabi et al. 2017; Fareed et al. 2017), for isolating fungi from plant tissues. The plant part plated for incubation is also an important element. Some parts are found to have more fungal endophytes than others, and again there is marked difference in the biosynthetic abilities of fungi based on their location within the plant body. Shubha and Srinivas (2017) performed screening tests on endophytes of *Cymbidium aloifolium* and found that root endophytes were most efficient in protease production, followed by endophytes of leaves and flowers. After successful isolation, the isolates are screened for protease production qualitatively and/or quantitatively. Further

selection is always based on quantitative assessment of enzyme production by the isolates (dos Santos Aguilar and Sato 2018).

10.6.2 Screening Methods

Screening methods involve testing whether the endophytic fungal candidate in question is capable of producing protease. The initial screening can be performed to simply assess the presence of protease synthesizing ability, whereas further investigation ascertains if the fungus is able to synthesize in quantities useful at the industrial level. The screening procedures have to be kept constant for all the isolates. Results of screening methods provide potential producers, often more capable than commercially used strains. Endophytic fungi isolated from different plants have been screened for protease production, and many workers have obtained positive results in screening (Table 10.3). Alberto et al. (2016) found an endophytic strain of *Diaporthe* sp. that had proteolytic activity comparable to the commercially used *Aspergillus oryzae*. Screening techniques also reveal the competence in protease production of endophytic isolates procured from the same plant. Patil et al. (2015) observed that among the endophytic assemblage comprising of species of *Aspergillus*, *Biosporus* and *Rhizoctonia*, *Biosporus* sp. showed maximum protease activity. Various strains of endophytic *Aspergillus*, such as *Aspergillus* sp. from *Alpinia calcarata* and *Aspergillus japonicus* from *Cymbidium aloifolium*, have been reported by many workers to have remarkable proteolytic ability (Sunitha et al. 2013; Shubha and Srinivas 2017). Some rare fungal endophytes like *Isaria* sp. isolated from *Calophyllum inophyllum* and *Stemphylium* sp. of *Eremophila longifolia* have demonstrated exceptional proteolytic activity (Sunitha et al. 2013; Zaferanloo et al. 2013). Likewise, common genera of fungal endophytes have also exhibited similar properties, for example, *Colletotrichum gloeosporioides* and *Trichoderma* spp.—endophytes of *Cymbidium aloifolium*— and *Phoma herbarum*, *Phoma* sp. and *Alternaria alternata* isolated from *Eremophila longifolia* (Zaferanloo et al. 2013; Shubha and Srinivas 2017). Many workers have reported strains of sterile fungi to have extraordinary potential in protease production. Screening methods can also reveal if the enzyme sources are resilient to temperature and pH fluctuations, indicating potential use in industrial applications.

10.6.2.1 Solid Plate Methods

Agar plate methods are the most popular among screening methods. They are based on the fungi's capability to utilize a polymeric nitrogen source by secreting proteolytic enzymes into the surrounding medium. It involves selection of appropriate medium composition along with a protein substrate. The fungal isolate is either directly grown on the medium or its culture broth is applied to see if it can digest the proteinaceous substrate. The digested protein gives a clear halo that is visible

Table 10.3 The functional attributes of endophytic fungi for protease production

Endophytic fungi	Host plant	Reference
Alternaria chlamydosporus, *Pestalotiopsis* sp., *Alternaria* sp., *Aspergillus* sp.	*Acanthus ilicifolius*, *Acrostichum aureum*	Maria et al. (2005)
Alternaria alternata, *Alternaria arborescens*, *Ascochytopsis vignae*, *Coniothyrium olivaceum*, *Coniothyrium* sp., *Diaporthe* sp., *Drechslera biseptata*, *Glomerella miyabeana*, *Gnomoniella* sp., *Helminthosporium velutinum*, *Leptosphaeria* sp., *Microsphaeropsis arundinis*, *Paraconiothyrium brasiliense*, *Phoma* sp., *Phoma glomerata*, *Pseudocercosporella* sp., *Septoria* sp., *Sirococcus clavigignenti*, *Coelomycetes* sp.	*Acer truncatum*	Sun et al. (2011)
Fusarium oxysporum	*Musa* sp.	Ng'ang'a et al. (2011)
Alternaria alternata, *Cladosporium* sp., *Leptosphaerulina* sp., *Nigrospora* sp., *Phoma herbarum*, *Phoma minima*, *Phoma moricola*, *Phoma* sp., *Stemphylium* sp.	*Eremophila longifolia*	Zaferanloo et al. (2013)
Colletotrichum crassipes, *Colletotrichum falcatum*, *Curvularia vermiformis*, *Drechslera hawaiiensis*, *Xylaria* sp.	*Coleus aromaticus*, *Adhatoda vasica*, *Lawsonia inermis*	Amirita et al. (2012)
Colletotrichum gloeosporioides	*Costus igneus*	
Acremonium terricola, *Aspergillus japonicus*, *Cladosporium cladosporioides*, *Cladosporium sphaerospermum*, *Fusarium lateritium*, *Nigrospora sphaerica*, *Penicillium aurantiogriseum*, *Pestalotiopsis guepinii*, *Phoma tropica*, *Phomopsis archeri*, *Tetraploa aristata*, *Xylaria* sp.	*Opuntia ficus-indica*	Bezerra et al. (2012)
Mortierella hyaline, *Paecilomyces variabilis*, *Penicillium* sp., *Penicillium* sp., *Talaromyces flavus*	*Potentilla fulgens*, *Osbeckia stellata*, *Osbeckia chinensis*, *Camellia caduca*	Bhagobaty and Joshi (2012)
Alternaria sp., *Aspergillus* sp., *Curvularia* sp., *Fusarium* sp., *Mucor* sp., *Nigrospora* sp., *Stemphylium* sp.	*Triticum turgidum*, *Zea mays*, *Gymnema sylvestre*	Patel et al. (2013)
Alternaria sp., *Aspergillus* sp., *Cladosporium* sp., *Colletotrichum falcatum*, *Colletotrichum* sp., *Fusarium solani*, *Fusicoccum* sp., *Isaria* sp., *Mycelia sterilia* sp., *Myrothecium* sp., *Pestalotiopsis disseminata*, *Xylaria* sp.	*Alpinia calcarata*, *Calophyllum inophyllum*, *Bixa orellana*, *Catharanthus roseus*	Sunitha et al. (2013)
Eutypella sp., *Fomitopsis, Phoma* sp., *Pleosporales* sp.	*Bacopa monnieri*	Katoch et al. (2014)
Penicillium citrinum, *Fusarium* sp.	*Hibiscus* sp.	Ahmad et al. (2014)

(continued)

Table 10.3 (continued)

Endophytic fungi	Host plant	Reference
Alternaria sp., *Bipolaris* sp., *Colletotrichum* sp., *Lasiodiplodia theobromae*, *Phoma herbarum*, *Schizophyllum commune*	*Piper hispidum*	Orlandelli et al. (2015)
Alternaria alternata	*Asclepias sinaica*	Fouda et al. (2015)
Penicillium funiculosum, *Trichoderma viride*	*Cardiospermum halicacabum*	Chathurdevi and Gowrie (2016)
Alternaria alternata, *Arthrinium phaeospermum*, *Aspergillus ochraceus*, *Cladosporium cladosporioides*,*Colletotrichum dematium*, *Curvularia* sp., *Drechslera* sp., *Fusarium solani*, *Penicillium frequentans*, *Pestalotiopsis glandicola*, *Pestalotiopsis microspora*	*Andrographis paniculata*, *Cryptostegia* sp., *Dalbergia latifolia*, *Eupatorium* sp.	Prathyusha et al. (2015)
Aspergillus sp., *Biosporus* sp., *Chaetomium* sp., *Cladosporium* sp., *Colletotrichum* sp., *Curvularia* sp., *Fusarium* sp., *Rhizoctonia* sp.	*Azadirachta indica*, *Citrus limon* *Datura stramonium*, *Gossypium hirsutum*, *Magnolia champaca*	Patil et al. (2015)
Diaporthe sp., *Saccharicola* sp., *Saccharicola* sp.	*Luehea divaricata*, *Saccharum* spp.	Alberto et al. (2016)
Alternaria alternata, *Cladosporium cladosporioides*, *Diaporthe* sp.	*Cupressus torulosa*	Rajput et al. (2016)
Aspergillus flavus, *Aspergillus niger*, *Aspergillus ochraceus* *Cladosporium cladosporioides*, *Fusarium semitectum*, *Fusarium* sp., *Monascus ruber*, *Penicillium citrinum*	*Bambusa* sp.	Gabres et al. (2016)
Aspergillus awamori, *Aspergillus* spp., *Colletotrichum siamense*, *Colletotrichum truncatum*, *Fusarium nematophilum*, *Nigrospora* sp., *Penicillium* sp., *Peniophora* sp.	*Viola odorata*	Katoch et al. (2017)
Beltrania rhombica, *Chaetomium* sp., *Colletotrichum acutatum*, *Colletotrichum* sp., *Corynespora cassiicola*, *Curvularia* sp., *Cylindrocladium* sp., *Fusarium* sp., *Glomerella cingulata*, *Nigrospora oryzae*, *Nodulisporium* sp., *Pestalotiopsis* sp., *Pestalotiopsis* sp., *Phoma* sp., *Phomopsis* sp., *Talaromyces* sp.	*Acacia leucophloea*, *Avicennia marina*, *Butea monosperma*	Thirunavukkarasu et al. (2017)
Acremonium sp., *Aspergillus carneus*, *Eupenicillium javanicum*, *Fusarium oxysporum*, *Sarocladium strictum*, *Sarocladium zeae*, *Trichoderma koningiopsis*, *Penicillium simplicissimum*, *Aspergillus ustus*	*Zea mays*, *Oryza sativa*	Potshangbam et al. (2017)

(continued)

Table 10.3 (continued)

Endophytic fungi	Host plant	Reference
Alternaria alternata, *Aspergillus japonicus*, *Aspergillus sydowii*, *Cladosporium* spp., *Colletotrichum gloeosporioides*, *Colletotrichum truncatum*, *Curvularia lunata*, *Fusarium oxysporum*, *Helminthosporium* spp., *Penicillium chrysogenum*, *Penicillium purpurogenum*, *Rhizoctonia* sp., *Talaromyces rotundus*, *Trichoderma* sp., *Xylaria* sp.	*Cymbidium aloifolium*	Shubha and Srinivas (2017)
Fusarium sp., *Gliocladium* sp., *Aspergillus* sp.	*Phoenix dactylifera*	Abdennabi et al. (2017)
Penicillium marneffei, *Aspergillus fumigatus*, *Penicillium viridicatum*, *Microsporum gypseum*, *Trichophyton tonsurans*	*Ziziphus nummularia*	Fareed et al. (2017)
Curvularia australiensis, *Alternaria citrimacularis*	*Aegle marmelos*	Mani et al. (2018)

unaided, or it can be enhanced by flooding the plate with various chemical solutions. The solid plate screening methods are the most simple and cheap techniques that do not require much skill. But these are only qualitative in nature and tell little or nothing about the endophytes' quantitative abilities to produce protease. A number of studies have utilized glucose yeast peptone agar (GYP-agar) medium amended with 0.4% gelatin (Maria et al. 2005; Sunitha et al. 2013; Fareed et al. 2017). Fungal blocks were incubated on the plate, and after the period of incubation, saturated ammonium sulphate solution was poured on the plates that precipitated the undigested gelatin and gave visible clear zones. Fouda and his team (2015) used the same protein substrate but used yeast-malt agar as the basal media and mercuric chloride as the indicator. Katoch and her team (2014) inoculated endophytic fungi on casein starch agar plates with 1% skimmed milk. In one study, fungi were allowed to grow on skim milk-agar plates, and the clear zones were enhanced with 10% tannic acid solution (Zaferanloo et al. 2013). Budiarto and his co-workers (2015) used PDA modified with 0.1% gelatin to grow fungi directly on this media. Trichloroacetic acid (TCA) has also been used as an indicator for precipitating unused protein substrate. Alberto et al. (2016) used Manachini solution with 0.5% gelatin as inducer for growing fungi and separated the culture broth by filtration. The broth was then placed in wells on media prepared with 10% gelatin, 10% skim milk and 2% agar-agar in citrate-phosphate buffer (pH 5.0). Commercial protease of *Aspergillus oryzae* was used as reference. After proper incubation, enzymatic activity was evaluated by measuring the size of the clear halo around the wells. Shubha and Srinivas (2017) devised a method for partially quantifying the proteolytic activity shown by fungi on solid plates. By measuring the zone of clearance and the diameter of fungal colony and by calculating the difference between these two, they obtained the enzyme index of respective fungal isolates, which is helpful for comparing their enzyme-synthesizing ability.

10.6.2.2 Liquid Culture Method

Spectroscopic studies involving growing the fungi in liquid media and then studying extracellular enzyme properties are more time-consuming than simple solid-media screening but carry several advantages. This quantitative method can detect infinitesimal proteolytic activity that might go undetected in agar plate assays, since spectroscopy is a sophisticated technique that is sensitive to even slight changes in optical density. Screening through liquid culture was performed by Patil et al. (2015). Filtered liquid culture broths of fungi were added to 1% casein solutions. Digestion of protein by enzyme was allowed for 1 h, followed by addition of 0.5 M trichloroacetic acid to stop the catalysis. The reaction mixtures were then centrifuged to remove precipitate, and absorption was read at 275 nm. The quantity of enzyme which liberated 1 μg of tyrosine under assay conditions was termed as one enzymatic unit. This procedure is suitable for carrying out screening and enzyme assay simultaneously.

10.6.2.3 Gel Dot Blot Method

Thirunavukkarasu et al. (2017) developed this method that allows rapid screening of a huge number of fungi for extracellular protease production as well as partial characterization of the protease. The method involves preparation of wells created onto the plate and acrylamide gel with gelatin as substrate. A composite gel is made with gel strips of varying pH—pH 5.0, pH 7.0 and pH 9.0. The lyophilized culture filtrates of fungi are spotted onto the gel and incubated for 8–10 h, followed by staining of the gel using 0.025% Coomassie Brilliant Blue. Commercially available alkaline and acidic proteases were used as controls by the authors. Enzymatic action is visualized as clear zones on the deep blue gel. This method uses Coomassie Brilliant Blue which is very sensitive to low protein concentrations, as low as 30 ng. This method is superior to usual agar plate and spectroscopic assays as:

- It is more accurate in detecting enzyme activity and low concentrations of protein.
- A large number of samples can be screened in a short time.
- Their range of optimum pH can be speculated simultaneously.

10.6.2.4 Molecular/Genomic screening

Bryant and her team (2009) approached molecular techniques for identifying protease synthesis genes, particularly subtilisin-like proteases (SLPs) in the fungus *Epichloë festucae*, an obligate endophyte found in many grass species. Using a combination of polymerase chain reaction (PCR), transcriptome and whole genome analysis, they predicted 15 different kinds of SLPs in the genome of the endophyte. Degenerate primers for sequence amplification were designed based on the

conserved SLP sequences in the evolution clade. The predicted subtilisin-like protease genes were identified in a genomic library from *Neotyphodium lolii*.

10.6.3 Understanding More About Protease Function and Synthesis Efficiency

10.6.3.1 Scale-Up Culture in Liquid Media

Selection of an appropriate media to produce elevated amounts of enzyme is an important task and might require several steps of trial and error. The media composition ought to be favourable for both fungal growth and enzyme secretion. In laboratory context, generally submerged cultivation is preferred over solid-state cultivation (Li et al. 2012b). Media that are typically used for luxurious growth of all kinds of filamentous fungi are applied and might be accompanied by some inducers for better production of extracellular enzyme. For harvesting secreted protease from filamentous fungi, most scientists have utilized potato dextrose broth (PDB) in scale-up cultures (Budiarto et al. 2015).

10.6.3.2 Extraction and Purification of the Protein

Purification process of enzyme can be single-step or multistep, depending upon the extent of purification demanded by its potential use. Therapeutic applications require highly purified enzymes in small amounts, whereas others like the detergent and food industry need crude enzymes in huge quantities. Methods of extraction and purification of the protease exploit some chemical and physical characteristics of the protein molecule, viz. its solubility, size, polarity, binding affinity, charge, etc.

The most elaborate method of protein extraction and purification from an endophytic fungus has been described by Budiarto and his co-workers (2015). Following large-scale culture in PDB, they implemented a three-step purification process for achieving maximum purification of the protease. The culture broth was centrifuged, and the filtered supernatant was saturated with 90% ammonium sulphate. The resulting precipitate of extracellular protein was resuspended in a buffer of 25 mM Tris-HCl (pH 7.4). The solution was then dialysed for 24 h at 4 °C in dialysis tubing and then applied on DEAE-Sepharose with the same buffer used previously. The column was eluted using gradient concentration of NaCl. Active fractions were applied onto a Sephadex SG-75 column with the same buffer. Finally, obtained active fractions were freeze-dried and resuspended in the tris-HCl buffer for further experiments. Estimation of protein content of the active fractions was done by Bicinchoninic Acid Protein Assay method. Activity of fractions at each purification step through plate assay was assessed by placing 0.5% agarose medium containing 0.2% of gelatine in Tris-HCl buffer on a Petri dish. Fractions from column chromatography were loaded into wells created onto the plate and incubated for 24 h. The

development of clear zone around the wells was detected by applying Coomassie Blue dissolved in a mixture of methanol, acetic acid and water, followed by destaining step to remove staining solution using destain solution made from methanol, acetic acid and water until the clear zone could be seen visually. This three-step purification process, involving first step of ammonium sulphate precipitation followed by two steps of ion-exchange chromatography and gel filtration, was also enacted by Meshram and his associates (2016).

10.6.3.3 Characterization of the Enzymes

Ascertaining the physicochemical properties of protease is essential to identify industrial sectors it might find use in. In order to biochemically characterize the enzyme, one must determine enzyme activity and study enzyme kinetics. Most authors have followed the method described by Kunitz (1947) with varied modifications to determine protease activity. It is based on monitoring spectrophotometrically the amount of tyrosine liberated through casein hydrolysis. In work done by Maria et al. (2005) and Budiarto et al. (2015), 1 ml enzyme filtrate was added to 1 ml 2% casein suspended in 100 mM phosphate buffer (pH 7.6). After 20 min of incubation at 35 ± 1 °C, the reaction was terminated by adding 3 ml of ice cold 0.306 M TCA. One millimetre of the clear TCA soluble extract was mixed with 5 ml 0.4 M Na_2CO_3 and 0.5 ml 1 N Folin-Ciocalteu reagent. The absorbance was measured at 660 nm/540 nm. One unit of protease activity was defined as 1 μmol of tyrosine released during catalysis per ml of reaction mixture per minute under the experimental conditions. Mayerhofer et al. (2015) quantified protease activity of culture broths of endophytic fungi using fluorescently labelled casein as substrate. Incubation was done in citrate-citric acid buffer ranging from pH 2.0 to 6.0. Proteolysis was studied by reading the excitation at 590 nm and emission of 645 nm. Fluorescence of each sample was calculated by subtracting uninoculated control values from the obtained sample values.

Determination of optimum conditions for protease activity and enzyme kinetics study are based on varying incubation parameters such as temperature, pH and concentrations of substrate. Budiarto et al. (2015) following the protocol of Zhang et al. (2010) created a temperature gradient of 20 to 90 °C and pH gradient using different buffer systems (citrate buffer for pH 4–6, Tris-HCl for pH 7–9, glycine-NaOH for pH 10–11). The value of K_m and V_{max} was determined based on the Lineweaver-Burk plot created by plotting the reciprocal of substrate concentration on the X-axis and reciprocal of the enzyme reaction velocity on the Y-axis by mixing crude enzyme with different concentration of casein ranging from 0.2% to 0.02%. They found protease-specific activity to be 0.091 IU/mg, optimum temperature as 60 °C and optimum pH 7 and K_m and V_{max} values of 0.183 mg/ml and 7.01 μg/min, respectively. Meshram et al. (2016) through similar experiments determined specific activity of their isolated protease from endophytic *Xylaria curta* to be 36.67 U/mg with an optimum activity at pH 8 and temperature 35 °C. They obtained K_m and V_{max} of 246 μM and 1.22 U/ml towards fibrin, 282 μM and 0.13 U/ml for plasmin, 298.2 μM

and 0.15 U/ml for streptokinase and 240.0 μM and 1.10 U/ml for fibrinogen, respectively.

Determination of molecular mass of the protein is either done through traditional technique of gelatin zymography or contemporary methods of mass spectrometry. Budiarto and his co-workers (2015) performed gelatin zymography for molecular mass determination by running the sample on 0.2% gelatin-containing gel electrophoresis. After complete run and separation of individual components, denatured protein was reactivated by incubation in 2.5% Triton X-100 for 40 min at 37 °C. Then the gel was stained with 0.05% Coomassie Blue and kept for 2 h. Removal of excess stain using destaining solution until clear band appeared on gel indicated protease activity. Interpolation deduced from linear logarithmic plot of relative molecular mass against R_f value of the protein band appearing on the gel gives the molecular mass of the protein. Through this method, they arrived at a molecular mass of 43 kDa on gelatin zymogram. Meshram and his team (2016) employed the sophisticated technique of MALDI-TOF (matrix-assisted laser desorption/ionization-time of flight) mass spectrometry for determining the molecular mass of their protease and found it to be a 33.76 kDa protein. Mass spectrometry assisted by MALDI-TOF is the most suitable modern method for molecular mass determination of protein that gives accurate results in a short span of time without much hassles of carrying out physical experiments.

A simple and fast way to determine the family or kind of protease is to investigate the effect of chemical inhibitors on protease activity. A number of inhibitors are used for this purpose, each one specifically inhibiting a particular class of protease as depicted below:

- Serine protease inhibitors—phenylmethylsulfonyl fluoride (PMSF) and Leupeptin
- Metalloprotease inhibitors—ethylenediaminetetraacetic acid (EDTA) and ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA)
- Cysteine protease inhibitors—tosyl phenylalanyl chloromethyl ketone (TPCK), iodoacetate, E-64 and Leupeptin
- Aspartic protease inhibitor—pepstatin A
- Threonine inhibitor—Leupeptin

By studying residual activity upon inhibition, Meshram et al. (2016) characterized their enzyme as a metalloprotease which was inhibited by EDTA and EGTA. Budiarto et al. (2015) identified theirs to be a serine protease as it was inhibited by PMSF. Characterization of protease reasserts their suitability for use in industrial settings where robustness and resilience are required over uncontrollable physical conditions that may lead to hostile temperatures, pH, presence of inhibitors and oxidizing agents. Proteases with broad range of activity and versatile applications are always coveted.

10.7 Industry Scale Production

10.7.1 Optimization

Under natural conditions without forcing any kind of manipulation, proteases produced by endophytic fungi are quite moderate in amount. To upscale their production to levels that are industrially sustainable, optimization of the fermentation process needs to be done. Protease production by endophytic fungi is affected by several intrinsic as well as extrinsic parameters. Intrinsic factors include the morphology and metabolic state of the fungal culture, while extrinsic factors consist of external media composition, temperature, pH, aeration, presence of inhibitors and inducers, competing species, etc. Process optimization has recently come under focus with regard to fungal endophytes, as they are increasingly finding use in industrial processes as manufacturers of bioactive products. Optimization studies shed light on the interactions between various factors affecting production, so that negative interactions can be avoided and positive interactions can be promoted. Finding the optimum fermentation conditions begins with fixing on the type of fermentation to be approached—solid or submerged. Parameters common to both types and exclusive to each type need to be assessed for establishing the most appropriate conditions for protease production with regard to a particular strain of endophytic fungi.

In the past few years, several researchers have performed optimization of protease production by endophytic fungal isolates. Maria et al. (2005) optimized the period of incubation best suited for *Pestalotiopsis* sp. in static culture using wheat bran seawater medium, observing fluctuations over 3, 6, 9, 12 and 15 days of culture. Zaferanloo and her co-workers (2013) assessed protease production by endophytes over a range of pH and incubation temperature and concluded that enzyme secretion was dominant at low pH and low temperatures. Comprehensive optimization of protease production by endophytic strain *Alternaria alternata* EL17 was accomplished by Zaferanloo and her team (2014). Regulation of fermentation process needs study of impact of changes in one parameter while keeping other parameters constant. Culture conditions that are economically viable need to be ascertained. The discrete parameters affecting enzyme production are briefly described below.

10.7.1.1 Incubation Period

Age of the culture affects enzyme production significantly since production of each metabolite is characteristic of a definite phase in the growth curve of a fungus. Optimum enzyme production can occur anytime between 24 h incubation to a week depending on the culture conditions and metabolic state of the fungus. Budiarto et al. (2015) noted that the early phase produced maximum harvest of protease from *Xylaria psidii*. Endophytic *Alternaria citrimacularis* and *Curvularia australiensis* both showed maximum enzyme secretion at 7th and 11th day which then remained

constant up to day 20 (Mani et al. 2018). Maria and her team (2005) made the observation that protease production reached peak on 6th day irrespective of pH of the culture solution. Though incubation period acts as a critical element, in many cases, it has been found that enzyme production by an organism is not growth associated (Sharma et al. 2017).

10.7.1.2 pH

pH of the culture medium is pivotal in determining the growth and morphology of the organism since cells are sensitive to the hydrogen ion concentration of the surrounding media (Rajput et al. 2016). pH of the media regulates all enzymatic reactions and transports across membrane, affecting chemiosmosis by proton motive force. Under optimum pH levels, the metabolic efficiency of a cell is highest, and consequently, its enzyme synthesis is also at its highest at this pH (Sharma et al. 2017). Optimum pH of culture conditions may or may not reflect the optimum pH of the protease. Mani et al. (2018) observed optimum pH for fermentation of endophytic *Alternaria citrimacularis* and *Curvularia australiensis* to be pH 7. Similar observation was made by Zaferanloo and team (2013) in case of *Phoma moricola*, *Nigrospora* sp., *Cladosporium* sp. and *Alternaria* spp. and in another work by Zaferanloo et al. (2014) in fermentation of *Alternaria alternata*. Zaferanloo and her co-workers (2013) reported *Stemphylium* sp. and *Phoma herbarum* to have maximum protease activity at the alkaline pH of 9, while *Phoma minima* had an acidic pH optimum of 5.5. Rajput et al. (2016) studied relative effect of different pH ranging from acidic to alkaline and found neutral pH range to be most suitable, followed by alkaline range and drop in activity at acidic pH range.

10.7.1.3 Temperature

Temperature is one of the most vital parameters that need to be controlled and kept in optimum range for maximum cell growth and enzyme synthesis. Fluctuations in incubation temperature can throw the organism into stress conditions and lead to the synthesis of obnoxious toxic metabolites, reducing the output of protease. Optimum temperature required by the fungus corresponds to its habit, whether it is psychrophilic, mesophilic or thermophilic. Most endophytic fungi produce maximum protease at the thermophilic range (37 °C) like *Leptosphaerulina* sp., *Phoma minima* and *Alternaria alternata* (Zaferanloo et al. 2013). Zaferanloo et al. (2014) also found their strain of endophytic *Alternaria alternata* to be most active at 37 °C. They discovered best production of protease in *Phoma herbarum* at 50 °C and in another species of *Phoma* at 9 °C.

10.7.1.4 Metal Ions

Various chemicals and metal ions have been reported to have modulatory effects on enzyme synthesis pathways. Some act as inducers, while others have inhibitory effects. Calcium ions are, in general, known to be inducers and help in stabilizing many enzymes by preventing conformational changes. This was confirmed by Meshram et al. (2016) as Ca^{2+} increased xylarinase activity. Proteolysis by this enzyme was found to decrease in presence of Cu^{2+} and Mn^{2+} and was completely inhibited by Zn^{2+} and Fe^{2+}.

10.7.1.5 Substrate

Selection of a substrate is perhaps the most important factor in making the enzyme production process commercially feasible. The culture media claims up to 30% of the cost of enzyme manufacture. Hence, it is important to use ingredients that maximize protease production while cost-cutting at the same time. Submerged fermentation allows amalgamation of different ingredients, each having positive upregulating effect on the process and giving the liberty of exclusively selecting each macro- and micronutrient. Solid-state fermentation allows the use of low-cost substrates from industrial and agricultural wastes. Solid wastes like wheat straw or barley, sugar cane bagasse, coffee pulp, grape wastes, copra paste, inert materials like resins of ionic exchange, acrolite or polyurethane foam have been applied for use as solid substrates for protease production. Gabres et al. (2016) investigated the proteolytic activity of endophytic fungi of bamboo leaves on the bamboo leaf litter through solid-state fermentation. They utilized bamboo leaves as a substrate with distilled water making moisture levels of 60–65%. Sometimes, two or more substrates are used in combination to elevate yield of protease. Substrate selection based on optimization must be verified for cost-effectiveness and regular supply and availability of the raw materials.

10.7.1.6 Carbon Source

Carbon is the element most abundantly required by any organism. Besides being a nutritional requirement, the carbon source also affects protease synthesis by having upregulating or downregulating activity. Various organic and inorganic sources of carbon have been investigated for their effect on proteolytic activity of endophytic fungi. Rajput et al. (2016) concluded that glucose was most effective in optimizing protease secretion, followed by maltose, sucrose, galactose and lactose. Zaferanloo et al. (2014) found the complex carbohydrates of soybean to be most effective, among starch, glucose, sucrose and maltose when used as a carbon source. Interestingly, some carbon sources inhibit protease synthesis through catabolic repression mechanism. In their absence, the protease has to play an additional role of providing carbon from amino acids. Conversely, protease activity declines when

the energy status of the cell is high and the cell has overabundance of carbon source. It is now known that the catabolite control protein (CcpA) is responsible for such regulation and acts as a signal for the repression in protease synthesis (Tehran et al. 2016). Hence, it is important to identify such sources of carbon to either avoid them in media composition or to adjust their concentrations to achieve desirable results.

10.7.1.7 Nitrogen Source

As important variable needed for growth and sustenance, nitrogen is preferable in diverse forms by each living being. Since nitrogen is requisite for amino acid and hence protein synthesis, adequate amounts of the form feasibly processable by the fungus need to be provided in the culture medium. Researchers have utilized a number of organic and inorganic, simple and complex forms of nitrogen to find the one that provides highest yield of protease. Zaferanloo et al. (2014) investigated impact of tryptone, yeast extract, casein, peptone and L-asparagine on protease production and found tryptone to give the most desirable results. Rajput and co-workers (2016) report yeast extract to be most suitable, followed by beef extract, peptone, ammonium nitrate, ammonium carbonate and urea.

10.7.1.8 Moisture Content

In case of solid-state fermentation, where the water availability is limited, it is necessary to provide the adequate amount of moisture for optimal growth and produce. Increased moisture content decreases porosity of the substrate, thereby reducing oxygen availability to the growing mycelia. As reduced porosity decreases gas exchange, temperature of the solid substrate rises, disturbing the ideal conditions of incubation. Low moisture content retards growth, decreases nutrient solubility and lowers the degree of swelling of substrate, all contributing to poor yield of enzyme. Optimized water levels in the substrate are necessary both for proper growth and fermentation as well as ease of product recovery (Sharma et al. 2017).

10.7.1.9 Particle Size of Substrate

Surface area available for growth of fungal mycelia in solid culture is important in determining enzyme synthesis rate. Smaller particles provide greater surface area for fungal hyphae to grow and attach to, facilitating nutrient exchange. But it may also lead to agglutination of the particles, resulting in decrease in aeration and diffusion. Larger particle size enhances diffusion but limit the surface area for growth. Hence, for optimum production of enzyme, a compromised particle size needs to be provided (Sharma et al. 2017).

10.7.1.10 Agitation and Aeration

In submerged fermentation, agitation and aeration of the liquid culture media are required for two reasons—for dissolving oxygen needed by the growing hyphae and for homogenization of mycelial mass, nutrients and products within the culture broth (dos Santos Aguilar and Sato 2018).

10.7.2 Systems of Fermentation

Fermentations performed at the laboratory scale are mostly submerged fermentation (Smf). Industries employ both submerged and solid-state fermentation (Ssf) for elevated levels of product formation. The difference between the two fermentation techniques lies in the availability of free water to the growing fungal filaments. Both systems have their own benefits and drawbacks, and choosing the appropriate technique is crucial for optimum product recovery.

10.7.2.1 Submerged Fermentation

This fermentation technique involves growing the fungal culture in liquid substratum with predefined composition. Submerged culturing allows greater control over incubation parameters, such as temperature, aeration and pH. Individual ingredients of the media can be adjusted according to demand of fermentation process. It has added advantage of ease of sterilization of media. Despite being cost-intensive, due to the benefits this process provides, submerged cultivation is preferred for protease synthesis where consistent production is required.

10.7.2.2 Solid-State Fermentation

Solid-state fermentation is the cultivation of filamentous fungi over solid material in the absence of any free liquid. Ssf is a promising technology that allows the use of agro-industrial wastes, carrying out both enzyme synthesis and degradation of waste material discarded by industries. Through ssf, many industrial residues have been put to good use, like cassava bagasse, sugarcane bagasse, sugar beet pulp/husk, orange bagasse, oil cakes, apple pomace, grape juice, grape seed, coffee husk, wheat bran, coir pith, etc., and have been used as raw materials for growing protease producing fungi (Bhargav et al. 2008). Ssf provides many advantages over smf and is considered more instinctive for fungi, since in nature filamentous fungi are found growing on solid substrates. Due to minimal amounts of available water, ssf results in formation of highly concentrated products that make the downstream processing quite effortless. Solid culture also deters bacterial contamination. Ssf is emerging as an economically and ecologically viable option that requires low capital investment,

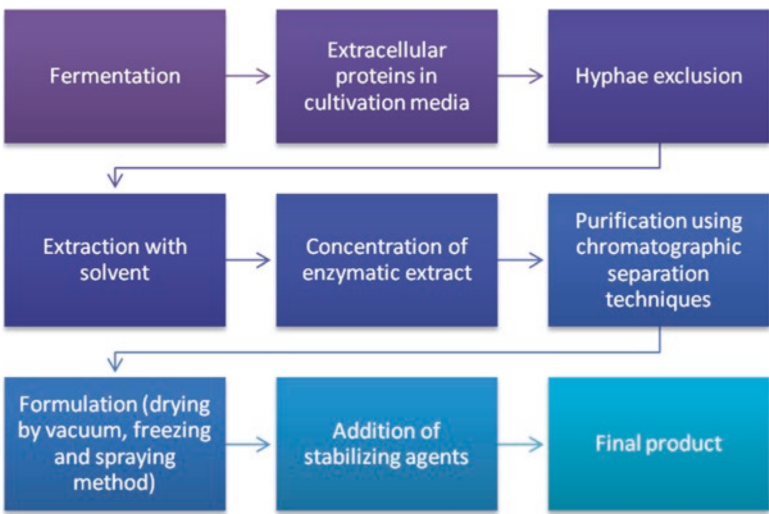


Fig. 10.4 Schematic diagram representing downstream processing of protease after fermentation

simpler machinery, low-energy input, use of cheap substrate and reduced catabolite repression and yields superior productivity and low wastewater output (Sharma et al. 2017).

10.7.2.3 Downstream Process

After completion of fermentation, the protease must be separated, concentrated and purified. This final step in commercial enzyme production is known as downstream processing or bioseparation. It can constitute up to 60% of the total production costs, excluding the charge incurred in purchase of raw materials. The downstream processing includes methods such as extraction, concentration, purification and stabilization and requires various chemical solvents, highly efficient machinery and skilled labour. The general scheme representing downstream processing for enzyme isolation is depicted in Fig. 10.4.

10.8 Attempts at Characterizing Proteases with Specific Industrial Use

Most studies involving proteolytic abilities of endophytic fungi have been confined to qualitative screening for enzyme production. Despite promising display of proteolytic activity by many endophytic strains, it would be superfluous to expect endophytic fungi and their proteases to be superior to the existing sources and enzymes. In this chapter, a few representative studies have been described where the

researchers have went beyond conventional approaches and established their discovered proteases as appealing solutions to lingering problems.

10.8.1 Detergent Industry

Among all commercial protease applications, detergent industries are the most dominant protease demanders. For this particular application, proteases need to have certain characteristics, like optimum activity at high and low temperatures and high pH, stability in presence of chelating and oxidizing agents, etc. Sources that secrete protease in huge amounts in cultivation media are sought after. Zaferanloo and her team (2013) studied physicochemical properties of protease produced by *Phoma herbarum* isolated from *Eremophila longifolia* and characterized this protease to be active at low temperatures and high pH. They suggest potential use of this protease in detergents for cold washing. Suggested disposition is through wax-coated granules to prevent inhalation of protease dust.

10.8.2 Food Industry

Rajput and his co-workers (2016) identified the optimum conditions for the protease production of endophytic isolate of *Alternaria alternata*, and their findings show that the endophyte possesses the ability to produce protease in a wide range of pH (3–12) and temperature (25–50 °C). The optimum temperature and pH for fermentation were noted to be 27 °C and pH 7, respectively. Deducing from these preliminary data, the authors suggest that the protease from *A. alternata* EL-17 can be applied to cheese-making and in milk clotting where the fermentation conditions are suitable for the activation of protease and its limited thermal tolerance ensures deactivation upon cooking. Zaferanloo et al. (2013) characterized protease of *Phoma* sp. as being most active at low pH and low temperature, making it a suitable candidate for use in food and confectionary.

10.8.3 Biomedical Sectors

A few workers have screened endophytes with the distinctive purpose of investigating their fibrinolytic activity and potential use in thrombolytic therapy. Naturally produced fibrinolytic agents can play a pivotal role in thrombolytic therapy which might be able to cure many heart-related diseases that are caused by accumulation of fibrin, the primary protein component of blood clot, in blood vessels forming a haemostatic plug or clot. Diseases such as myocardial infarction, high blood pressure, valvular heart disease and ischaemic heart diseases call for such thrombolytic

therapy that is cost-effective with fewer side effects (Meshram et al. 2016). Significant contribution in this sector was made by Wu et al. (2009) who described a novel fibrinolytic enzyme from the endophyte *Fusarium* sp. CPCC 480097. The endophyte was isolated from chrysanthemum stems, and protease produced by it showed excellent fibrinolytic activity. The protease was studied to be a 28 kDa protein, with an isoelectric point of 8.1 and maximum fibrinolysis at 45 °C and pH 8.5.

Li et al. (2007) tested endophytic isolates of *Clonostachys* sp., *Cladosporium* sp., *Fusarium* sp. BLB and *Verticillium* sp. from *Trachelospermum jasminoides* for in vitro thrombolytic, fibrinolytic, fibrinogenolytic and anticoagulant activity and found positive results. Another endophyte isolated from *Hibiscus* leaves was screened for fibrinolytic activity after observing its capability of utilizing skim-milk agar. Ahmad et al. (2014) tested the fibrinolytic activity of two endophytic fungi, identified as *Penicillium citrinum* and *Fusarium* sp. through fibrin plate screening and found both of these to possess positive fibrinolytic protease activity.

The most remarkable work was done by Meshram and his team (2016) in their description of a bifunctional metalloprotease produced by *Xylaria curta* that they have named xylarinase. The protease possesses superlative plasmin-like ability of hydrolysing fibrin, independent of plasminogen. Xylarinase can hydrolyse both fibrin and its precursor. Dose-dependent dissolution of thrombus revealed minimum amount of 50 μl of protease required. This suggested better efficacy than plasmin. N-terminal sequencing of the protein revealed it to be a novel protease. Its molecular mass was determined at 33.76 kDa. The mechanism of its action is postulated to involve blocking the activation of blood clotting cascade by suppressing the thrombin pathway. It has been shown to have no hemorrhagic effect in vitro. As stated by the authors, Xylarinase stands out as a prospective candidate in producing therapeutic agents, as evidenced in preclinical studies in thrombolytic therapy.

10.8.4 Agri-industries

10.8.4.1 Litter Degradation

The role of endophytes in litter degradation of their associated senescent host tissues had been postulated for many years. Two unrelated groups of researchers confirmed this hypothesis through their work. Kumaresan and Suryanarayanan (2002) studied the role of foliar endophytes in mangrove litter degradation. The endophytic assemblage of intact as well as senescent leaves in both wet and dry fallen conditions was investigated, and the enzyme activity of the isolated endophytes was tested. *Glomerella* sp. MG108 was found to be an active producer of protease and many other hydrolytic enzymes that degrade the plant litter. Endophytic community, in a whole, possesses the complete enzyme array to degrade leaf litter, and protease is an important part of that conglomeration of hydrolytic enzymes. The authors suggest that future studies involving the role of endophytes in agri-industry waste degradation is worthwhile and holds promising results.

Sun et al. (2011) also concluded through similar findings that the degrading enzymes of endophytes of *Acer truncatum* had a significant role to play in litter degradation. Gabres et al. (2016) studied the digestion of bamboo leaf litter by native endophytic fungi. The fungi were able to lower the protein content of the litter through proteolysis. The workers noticed an increased amount of fibre in the fermented litter that could have been formed due to increased tannin-protein complex production and suggest the use of such leaves as fodder for horses, as increased fibre imparts greater stamina through improved digestion and peristalsis. The authors foresee that these endophytes have the potential for use in industries as sources of protease. Orlandelli et al. (2015) vouch for the use of agro-industrial wastes and other such waste products as substrates for production of proteases by endophytic fungi. They found that endophytic fungi from *Piper hispidum* could efficiently produce protease in media consisting of rice flour and soy flour, both by-products of agro-industries. This can provide the industries with cost-effective raw materials that are both cheap and abundantly available.

10.8.4.2 Protease in Bio-control Tool Designing

While insecticides have been the primary dependence to protect commercial crops, it has not gone unnoticed that they cause severe deterioration of the environment and are also detrimental to all forms of life. Keeping in mind these facts and the increasing instances of insecticide resistance in previously susceptible pests, scientists have been working on devising safer options of biological control (Kour et al. 2017). In this front, Bensaci et al. (2015) observed that the endophytic fungus *Cladosporium oxysporum* isolated from *Euphorbia bupleuroides* subsp. *luteola* can be effectively used to control the black bean aphid (*Aphis fabae*) through formulations that contain protease from the endophyte. These genera include some species that are natural entomopathogens. Finding endophytic forms of this natural entomopathogen increases the chances of obtaining biocontrol formulations that are fast-effective and stable. The authors created invert emulsions of conidial suspensions of the fungus, characterized by a discontinuous aqueous phase within a continuous oily phase. Spray treatment of the aphids by the invert emulsions resulted in more than 90% mortality within half an hour. The fungus could effectively germinate and invade the cuticle of the aphid. Proteolytic activity of the fungus is a predominant factor in this pathogenesis. The enzyme is responsible for degradation of the cuticle of insect and invasion of the fungal hyphae; its production and activity were observed to be consistent with the aphicidal activity. Authors note that the fungus and its proteolytic formulations can be successfully exploited in biological control programmes for several aphids in semiarid and arid agricultural ecosystems. Other studies involving aphicidal activity of endophytic counterparts of natural entomopathogenic fungi have demonstrated that endophytic fungi are more efficient in producing proteases that increase chances of successful colonization inside the host insect.

In a similar vein, Potshangbam and her co-workers (2017) tested endophytic fungal isolates for enzyme production and inhibition of pathogens in the quest to devise a bio-control agent. They also studied their growth parameters in different environmental conditions that could reflect their dynamic living conditions inside host plants. Their results conclude that protease is a key enzyme that decides their suitability as a biocontrol agent as it provides protection against insect pests. The study found that the endophytic fungal isolates that produced highest amounts of protease were able to inhibit pathogens and also their colonization following artificial inoculation was successful. The authors suggest that such endophytic fungi are promising bio-resource agents.

10.8.5 Bioremediation

A rather prodigious work concerning practical application of endophytic protease was done by Russell and his associates (2011). Endophytic fungi were isolated from rainforest trees and screened for their ability to degrade the polyester polyurethane (PUR). PUR is a widely used polymer that has responded to a few attempts of biodegradation. Screening for positive-degrading ability was done by inoculating the endophytic fungal isolates in media containing PUR as the sole carbon source. Two strains of *Pestalotiopsis microspora* were found to possess excellent ability to degrade PUR even under anaerobic conditions. The enzyme responsible for degradation was identified as a diffusible secreted protein obtained by filtration through a 0.22 μm membrane that was denatured when the temperature was raised to 98 °C. The enzyme was further characterized to be a serine protease when it was observed that serine hydrolase-specific inhibitor PMSF inhibited its activity. The culture filtrates containing the enzyme were capable of clearing the polymer to a huge extent within a short period of time. Authors conjecture that endophytes are a useful bioresource and these enzymes from endophytes can be effectively utilized in bioremediation programs. They also suggest screening of endophytes for finding out degraders of other recalcitrant polymers that have been polluting the ecosystem.

10.9 Conclusion and Future Prospects

Growth of civilization has always depended on searching for newer and better resources. Focus is now on sustainable growth that aims at overall well-being of the planet. For adopting a carbon-neutral mode of development, enhancement of existing products and processes for increased efficiency and rational systems of waste management are required. Endophytic fungi as protease producers are advocates of sustainable system. With a limited number of studies, their modus operandi has been substantiated to be more efficacious than other traditional sources of proteases.

Novel proteases have been extracted from them that have the potential to function in challenging areas and provide solution to lingering problems ranging from the field of therapeutics to bioremediation. These primary metabolites are promising substitutes to harmful inorganic chemicals and secondary metabolites in industrial processes. This emerging field of research welcomes more, in-depth studies that will enable us to delve into the arsenal of metabolites produced by endophytic fungi and their applications on a broad scale in the practical world.

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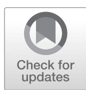
Chapter 11
Fungal Lipases: Versatile Tools for White Biotechnology



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Abstract Lipases (EC 3.1.1.3) catalyze the hydrolysis of triacylglycerols and the synthesis of esters from glycerol and long-chain fatty acids. They are also effective in transesterification in the absence of water, acidolysis, alcoholysis, interesterification, and aminolysis reactions. These enzymes also exhibit the phenomenon of interfacial activation. In general, industrial lipases are produced from wild and recombinant microorganisms obtained from heterologous expression. In this chapter, we are describing general properties, differences between lipases and esterases, diversification of lipase families, and structural and kinetic aspects. Generally, hydrophobic interaction steps are described to purify and immobilize lipases. The most important application is their addition to detergents, which are mainly used in household industrial laundry and dishwashers. Beyond, these catalysts are used in pharmaceutical, pulp and paper, chemical, textile industries, food processing, biodiesel production, and many others. Currently, the biotechnological potential of lipases is making them gain enormous attention in the white biotechnology.

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11.1 Introduction

People have been using enzymes for different purposes starting from ancient civilizations. Today, nearly 4000 enzymes are known, and among them, nearly 200 are commercially used. At least 75% of all industrial enzymes are hydrolytic, and the majority of them are of microbial origins, such as bacteria, yeasts, or fungi (Andualema and Gessesse 2012). Currently microbial enzymes cover 90% of the global market. The three major global enzyme producers (Novozymes, DuPont, DSM) are predominantly located in Europe and Asia and account for more than 75% of the global enzyme business. Novozymes is the only company present in all enzyme markets, showing Novozymes' quasi-exclusive dedication to the development of new commercial enzymes. Today, this company represents 45% to 50% of the enzyme market (Fig. 11.1). Other companies in Europe that also have excelled in the production and commercialization of new enzymes are AB Enzymes, BASF, Chr. Hansen, Kerry, and Soufflet Biotechnologies. Besides these companies in the enzyme market worldwide, Japan has also established itself in this market as emerging strong suppliers of enzymes like Ajinomoto, Amano, Nagase, and Shin Nihon. Finally, other countries such as China and India have appeared in the enzyme market, developing numerous commercial enzymes that allow supplying not only the domestic market of these countries but also the external market (Guerrand 2017).

Lipase is the common name for a group of enzymes belonging to the class of hydrolases (EC 3.1) that catalyze the hydrolysis of ester bonds (EC 3.1.1). They are carboxylesterases (carboxyl ester hydrolases or carboxylic ester hydrolases) that include esterases (EC 3.1.1.1) and lipases (EC 3.1.1.3) which are widely distributed in nature. Some authors consider lipolytic enzymes as other hydrolases that catalyze acylglycerols, such as the cutinases (EC 3.1.1.74) that hydrolyze the ester bonds of the cutin, a plant polymer, and the phospholipases A and B (Fojan et al. 2000; Sarmah et al. 2018). These enzymes are extremely versatile and highly efficient

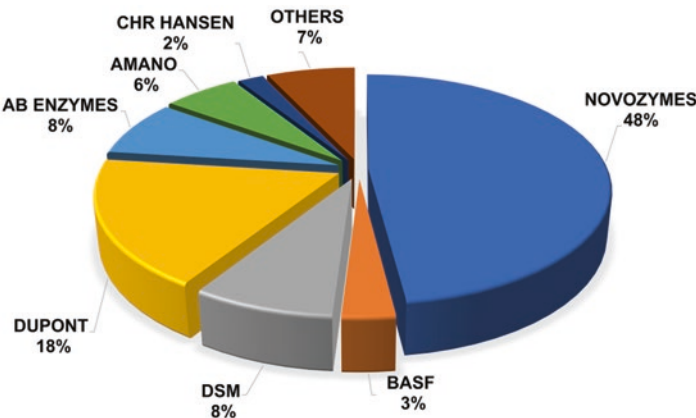

Fig. 11.1 Industrial enzyme market share, estimation by company (2015)

biocatalysts together with esterases and proteases. They play an important physiological role because in the presence of water they convert triacylglycerols to more polar forms like diacylglycerol, monoacylglycerol, free fatty acids, and glycerol. They are valuable biocatalysts because they act under mild conditions, are highly stable in organic solvents, show broad substrate specificity, and usually show high regio- and/or stereoselectivity in catalysis (Javed et al. 2018).

Lipases differ with respect to their origin and kinetic properties. Generally, lipases do not require cofactors, act in a wide pH range, are stable at high temperatures, and show high specificity and properties of regio-, chemo- and enantioselectivity that make them highly applicable in industrial processes (Hasan et al. 2006; Sarmah et al. 2018; Singh and Mukhopadhyay 2012; Villeneuve et al. 2000). These enzymes can be produced by many microorganisms and higher eukaryotes. The biodiversity described for lipases are: bacteria (45%), fungi (21%), animals (18%), plants (11%), and algae (3%) (Sarmah et al. 2018). Lipases from microorganism obtained by fermentation are preferable to those of animal sources and plants because they have shortened generation time, high yield of substrate conversion into product, regular supply due to the absence of seasonal changes, rapid growth of microbes producing enzymes on cheap cost-effective media, great variety of catalytic activities, and simplicity in the genetic manipulation and in cultivation conditions. Therefore, they are more viable from an economic and industrial point of view (Singh and Mukhopadhyay 2012).

Several genera of microorganisms can be used to produce lipases, such as fungi of the genera *Trichosporon*, *Botrytis*, *Pichia*, *Fusarium*, *Aspergillus*, *Mucor*, *Rhizopus*, *Penicillium* and *Geotrichum*; yeasts of the genera *Tulopis* and *Candida*; and bacteria of the genera *Streptomyces*, *Chromobacterium*, *Pseudomonas*, *Bacillus*, *Enterococcus*, and *Staphylococcus* (Faber 2004; Hasan et al. 2006; Javed et al. 2018; Sahay et al. 2017; Sarmah et al. 2018; Saxena et al. 2016; Singh and Mukhopadhyay 2012; Suman et al. 2015; Yadav 2015; Yadav et al. 2016, 2018a).

The utility of microbial lipase in trade and research is the result of its physiological and physical properties:

- Large amounts of purified lipase may become available, facilitating mass production. Lipases are active under environmental conditions, and the energy expenditure required conducting reactions at high levels of temperature and pressure is eliminated which reduces the destruction of labile reactants and products.
- Thermophilic microorganisms and enzymes stable at high temperatures and adverse chemical environments are of advantage in industrial uses.
- Due to the specificity of enzymes, unwanted side products that normally appear in the waste stream are reduced or eliminated.
- The use of enzymes can decrease the side reactions and postreaction separation problems.
- Processes catalyzed by lipase also offer cost-effectiveness, in comparison to the traditional downstream processing.
- Lipases remain active in organic solvents in their industrial applicability.

- When immobilized lipases are used under typical “industrial” conditions, reactor temperatures as high as 70 °C are possible for prolonged periods.

11.2 Classifications of True Lipases and Carboxylesterases

True lipases prefer highly hydrophobic substrates, which are insoluble in water and tend to form aggregates like oils and fats containing triacylglycerols with long acylglycerol chains (≥10 carbon atoms) (Bornscheuer 2002; Fojan et al. 2000; Giraldo et al. 2007). For this reason, often, the activity of true lipase is directly correlated with the substrate area and not with the substrate concentration (Cernia et al. 2002; Laszlo and Evans 2007). On the other hand, esterases have the ability to hydrolyze only short acylglycerol chains (<10 carbon atoms), and the enzymatic activity is restricted to the hydrolysis of ester bonds in water-soluble substrates and generally shows promiscuous activity with alcohol or acid moiety (Bier 1955; Bornscheuer 2002; Brockman 1984; Fojan et al. 2000; Salameh and Wiegel 2007).

Lipases have considerable levels of activity and stability in nonaqueous systems different from many other enzymes. The catalysis of lipases occurs at a lipid-water interface, where the substrate generally forms a balance between the monomeric, miscellaneous, and emulsified states. This characteristic makes possible for most lipases to perform a phenomenon known as interfacial activation. The interfacial activation was described by Holwerda et al. (1936) and Schonheyder and Volqvartz (1945). Through the measure of the pancreatic lipase activity using tricaproin as the substrate, the authors observed that the hydrolysis was increased when the concentration of the substrate exceeded the solubility limit. Sarda and Desnuelle (1958) observed that esterases were active only on molecular dispersed substrates, whereas lipases constituted a special class of esterases that showed greater activity on substrates forming aggregates. Thus, it was proposed that the phenomenon of interfacial activation would be a characteristic of lipases that could distinguish them from other esterases. However, it was later discovered that not all the lipases have the interfacial activation, then the classification based on the length of carbon chain is broader.

Other approach to differentiate lipases from esterases has also been made by the difference in the preferential specificity of this class of enzymes. This specificity can be for the substrate, positional, selectivity-type, and stereospecificity. In the case of the substrate, it is based on the difference of hydrolysis rates between triacylglycerols, diacylglycerols, and monoacylglycerols catalyzed by the same enzyme or enzymes purified from the same source. In positional or regioselectivity, the enzyme has preferential hydrolysis of primary, secondary, and tertiary esters or nonspecific hydrolysis, releasing fatty acids from the three positions. In the specificity and selectivity-type, the enzyme has preference for specific fatty acids, mainly regarding chain length and number of unsaturations. Finally, in relation to stereospecificity, the enzyme discriminates between enantiomers in racemic substrates, but it can be found in the combination of the types previously mentioned or even the absence of specificity (Sarmah et al. 2018). A summary of the differences between esterases and lipases is shown in Table 11.1.

Table 11.1 Differences between esterases and lipases

Characteristic	Esterase	Lipase
Preferred substrates	Triglycerides with <10 carbon atoms (e.g., tributyrin), simple esters (e.g., ethyl acetate)	Triglycerides with ≥10 carbon atoms (e.g., triolein), secondary alcohols (e.g., 2-propanol)
Substrate solubility	High	Very low
Amount of nonpolar amino acids on active site	Low	High
Interfacial activation	No	Yes
Presence of a "lid"	No	Majority
Enantioselectivity	High to zero	Usually high
Organic solvent stability	High to low	High

11.3 Types of Reactions of Lipase

The hydrolysis reaction of lipases is reversible, and in the presence of lower amounts of water, and often in the presence of organic solvents, they are effective catalysts in the ester synthesis by esterification. These basic processes of hydrolysis and esterification may be combined in a sequential pattern to give rise to a group of trans-esterification reactions. Depending on the starting substrate, the reaction is called acidolysis (when the acyl group is displaced between an ester and a carboxylic acid), alcoholysis (between an ester and an alcohol), or interesterification (between two esters) without any formation or consumption of water, besides other synthetic processes, such as aminolysis (amide synthesis) and lactonization (intramolecular esterification) (Fig.11.2).

11.4 Lipolytic Families

The lipolytic enzymes are classified as serine hydrolases due to the inhibition by diethyl *p*-nitrophenol phosphate. The mechanism of action is based on the triad of amino acids serine-histidine-aspartate/glutamate in the active site (Javed et al. 2018). Despite the structural similarity, these enzymes do not share any sequence similarity or do not operate under similar substrates, neither use the same nucleophilic site. However, they preserve the structural arrangement of residues present in the active site, suggesting a possible evolution from a common ancestor (Ollis et al. 1992).

The three-dimensional structure of lipases and esterases shows the characteristic α-/β-hydrolase fold (Ollis et al. 1992) and a definite order of α-helices and β-sheets. The α/β hydrolase fold domain is found in a number of functionally different enzymes that are capable of hydrolyzing substrates with different characteristics. For example, this superfamily includes proteases, lipases, esterases, dehalogenases, peroxidases, and epoxide hydrolases, and it is one of the most common protein folds found in nature (Hotelier et al. 2010; Nardini and Dijkstra 1999; Ollis et al. 1992;

Hydrolysis and esterification

$$R_1C(=O)O{-}R_2 + H_2O \underset{}{\overset{\text{Enzyme}}{\rightleftharpoons}} R_1C(=O)OH + R_2{-}OH$$

Transesterifications

Acidolysis

$$R_1C(=O)O{-}R_2 + R_3C(=O)OH \xrightarrow{\text{Enzyme}} R_3C(=O)O{-}R_2 + R_1C(=O)OH$$

Alcoholysis

$$R_1C(=O)O{-}R_2 + R_3{-}OH \xrightarrow{\text{Enzyme}} R_1C(=O)O{-}R_3 + R_2{-}OH$$

Interesterification

$$R_1C(=O)O{-}R_2 + R_3C(=O)O{-}R_2 \xrightarrow{\text{Enzyme}} R_1C(=O)O{-}R_4 + R_3C(=O)O{-}R_2$$

Aminolysis

$$R_1C(=O)O{-}R_2 + R_3{-}NH_2 \xrightarrow{\text{Enzyme}} R_1C(=O)NH{-}R_3 + R_2{-}OH$$

Fig. 11.2 Reactions catalyzed by lipase

Singh et al. 2016; Yadav et al. 2018b). Considering the difficulty in classifying lipases based on their mechanism of reaction, Arpigny and Jaeger (1999) proposed a classification system based on the sequence similarity of various enzymes with the lipase from *Pseudomonas aeruginosa*. The architecture of domains shared by lipolytic enzymes allowed their classification into eight different families based on their conserved amino acid sequences and on the catalytic properties of each enzyme (Arpigny and Jaeger 1999). Hausmann and Jaeger (2010) reformulated some characteristics of the family members of microbial lipases based on the discovery of new lipases and esterases and the resolution of three-dimensional structures by crystallography.

- *Family I* (triacylglycerol lipases) is the largest one and comprises the "true" lipases that have interfacial activation and the presence of a "lid" (this family is further divided into six subfamilies).

  The *families II–VIII* comprise the esterases (carboxylesterases) and other lipases:

- *Family II* (GDSL family) does not exhibit the conventional pentapeptide Gly-X-Ser-X-Gly but rather display a Gly-Asp-Ser-(Leu) [GDS(L)] motif containing

the active site serine residue. In these proteins, this important residue lies much closer to the N-terminus than in other lipolytic enzymes.

- *Family III* includes psychrophilic extracellular lipases from *Moraxella* sp. and various species of *Streptomyces*.
- *Family IV* displays a remarkable amino acid sequence similar to the mammalian HSL (hormone-sensitive lipase family).
- *Family V* also comes from psychrophilic and mesophilic bacteria and shares significant amino acid sequence similarities (20–25%) with several non-lipolytic bacterial enzymes.
- *Family VI* has the smallest carboxylesterases known with molecular mass in the range 23–26 kDa.
- *Family VII* includes large esterases (50–65 kDa), with sequence similarity with eukaryotic esterases of the intestine and with carboxylesterases of the liver.
- *Family VIII* does not present the typical α/β hydrolases structure but shows a remarkable similarity to several class C β-lactamases (Arpigny and Jaeger 1999).

Nowadays in the era of genomics and bioinformatics, a number of new lipases have been discovered that do not fit in the existing criteria of classification. Therefore, a comprehensive approach is needed to delimitate a wider criterion to the classification of lipases (Arpigny and Jaeger 1999; Eggert et al. 2001; Javed et al. 2018).

11.5 Lipase Structure

Studies on lipase three-dimensional structure showed that this group of enzymes contains a canonical α/β hydrolase fold, a motif shared with many esterases and peptidases. This motif consists of eight β-strands surrounded by six α-helixes (Fig. 11.3), although it was reported that the lipase from *Bacillus subtilis* lacked the β1 and β2 strands in the canonical fold (Eggert et al. 2001). As cited, lipases and esterases contain a pentapeptide Gly-X-Ser-X-Gly, which X may be any amino acid residue and Ser residue in the middle of the sequence forming a γ-like turn between

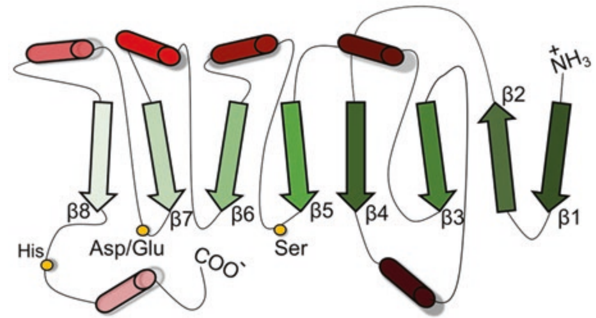

Fig. 11.3 Structure of α-/β-hydrolase motif in lipase. The amino acid residues that constitute the catalytic triad are represented in yellow dots

Fig. 11.4 Hypothetical structure of a hydrophobic binding pocket, or "tunnel," in a lipase active site. The regioselectivity of an unsaturated acyl chain is explained by the shape of that "tunnel," while the other two acyl chains will not interact with the ligand-binding site, being oriented to the solvent

β5 and the following α-helice (Mala and Takeuchi 2008). However, Akoh et al. (2004) have described a subclass of lipolytic enzymes which possess a distinct sequence from the Gly-X-Ser-X-Gly, named Gly-Asp-Ser-Leu. Both serines previously described is part of the active site with His and Asp/Glu residues, which forms a catalytic triad comparable to those of serine proteases (Brady et al. 1990; Jaeger et al. 1999). Other three-dimensional studies have shown the existence of lipid binding-pockets next to the catalytic triad, sometimes referred as a "tunnel" that connects the catalytic triad to the lipase core (Cygler et al. 1994; Holmquist 1998; Norin et al. 1994; Pleiss et al. 1998). The shape and dimensions of this "tunnel" might be associated with regioselectivity and enantioselectivity of a lipase (Schmitt et al. 2002), explaining why some lipases are capable of hydrolyzing one, two, or all the acyl chains or the selective hydrolysis of unsaturated or saturated chains in a triglyceride (Fig. 11.4).

Studies with *Candida antarctica* lipase B binding ligands to long acyl ester bonds showed that substrate-binding pocket is an elliptical and steep funnel of 9.5 × 4.5 A (Fig. 11.5). At the bottom of this funnel, there is a hydrophilic zone formed by the catalytic serine and aspartate which reaches to the C4 of the acyl chain. Up to C7 to C13, the binding site becomes hydrophobic constituted by valine, leucine, and isoleucine residues till the end of the "tunnel", which turns into the hydrophobic surface of the enzyme. Water molecules are necessary to hydrolyze the acyl-enzyme complex; however, the existence of a hydrophobic surface would limit the access to the active site. Then, it is assumed that water molecules are trapped in the hydrophilic zone present at the "tunnel" described earlier. A favorable water-binding site is present at the bottom of the "tunnel" structure in *Mucor miehei* opposed to a hydrophobic binding pocket for the acyl chain. Recent studies are providing mechanisms to modify this structure to enhance synthesis of acyl-specific groups with biotechnological value (Laguerre et al. 2017; Silveira et al. 2017).

In the catalytic triad (Fig. 11.6), without any substrate, the carboxylated residue (Asp or Glu) makes a hydrogen bond with a nitrogen in the His ring, and the other

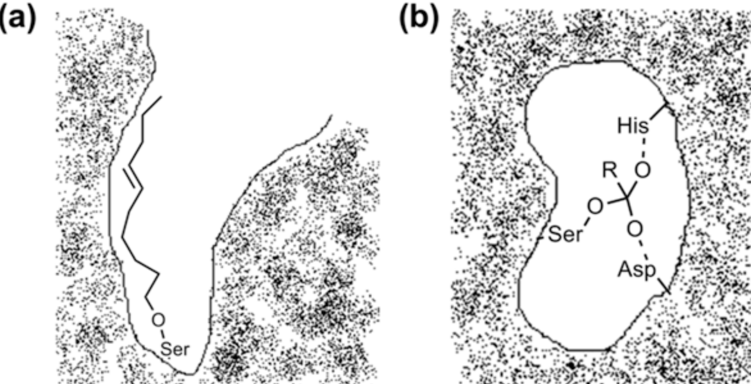

Fig. 11.5 Hydrophobic binding pocket (**a**) interacting with the long acyl chain present in a triglyceride and hydrophilic zone (**b**) positioning the ester bonds for hydrolysis, present in the active site of *C. antarctica* lipase B. (Modified from Pleiss et al. (1998))

Fig. 11.6 A schematic demonstration of the catalytic triad arrangement in lipase active site without substrate

nitrogen of the ring is positioned in such a way to make a hydrogen bond with the hydroxyl group of Ser (Jaeger and Reetz 1998). To explain how the catalytic triad works in the presence of a substrate, the steps of hydrolysis on triacylglycerol will be subsequently schematized (Fig. 11.7), but it should be emphasized that the esterification and transesterification occur in the same scheme as well as in reverse steps. The reaction begins with a nucleophilic attack by Ser hydroxyl on the carbonyl carbon of the lipid ester bound. A tetrahedral intermediate in which -O is stabilized by two -NH groups from His ring is formed. This promotes the protonation of the imidazole ring which is stabilized by the negative charge of Asp/Glu residue. At some moment, the hydrogen from His ring is donated to the -O which was stabilizing the structure, resulting in a free diacylglycerol. The fatty acid chain remains covalently linked to the enzyme Ser and stabilized by the hydrogen bound with Asp residue; this complex is called the acyl-enzyme (Bornscheuer 2002; Jaeger et al. 1999). A water molecule is activated by the His ring, promoting a hydroxyl ion nucleophilic attack at the carbonyl carbon atom of the acyl-enzyme complex. The

Fig. 11.7 Mechanism of lipase/esterase catalysis. (1) Catalytic site. (2) Lipid binding and activation of the nucleophilic serine residue with the formation of the tetrahedral intermediate and stabilization of O- by the interaction with two peptidic NH groups. (3) The intermediate acyl-enzyme is formed and undergoes nucleophilic attack by a molecule of water (hydrolysis) or another acceptor (transesterification). (4) Release of product and the catalytic site is restored

second tetrahedral complex is formed, but this time the His ring donates a hydrogen (previously provided by water) to the -O of Ser, which destabilizes the covalent bound with the fatty acid chain, releasing it and recovering the initial state of the enzyme (Bornscheuer 2002; Jaeger et al. 1999).

One particular aspect of lipase activity, differing from esterase and other enzymes, is that substrates are not soluble in aqueous environment beyond a certain concentration, which is called critical micelle concentration (CMC). It was observed that lipase activity was enhanced on lipid substrates beyond their CMC (Guerra et al. 2011), *i.e.*, when a lipid reaches the CMC, the system builds micelles and interfaces; highly hydrophobic structures are isolated from water. Since then, it was widely known that the presence of micelles or interface is important to activate the lipase (Hasan et al. 2006; Sharma et al. 2001). The reason was obtained through three-dimensional studies which discovered the existence of an α-helix that acts as a "lid" covering the catalytic amino acid residues (Jaeger et al. 1999; Lotti and Alberghina 2007).

The “lid” is not present in every lipase, but when it is, this structure covers the catalytic site making it inaccessible to substrates. Some lipases might have multiple “lid” domains (Khan et al. 2017). The “lid” of lipase is an amphipathic structure; the existence of a hydrophobic face was observed, which is buried within the catalytic site in aqueous solutions. When an interface of lipid, or micelle, is encountered, the hydrophobic face exposes itself to the substrate allowing it to enter the active site (Cygler and Schrag 1997). It has also been found that the open/closed “lid” conformations and modifications in its amino acid sequence interfere in lipase temperature and solvent stabilities (Khan et al. 2017; Maiangwa et al. 2017). Therefore, the “lid” is an important structure responsible for lipase’s amphipathic property, specificity, activity, and stability in reaction systems.

Each lipase has a different “lid” mobility degree. In some cases, there are apparently two states, open and closed, with energy levels significantly lower than the transition states. Many possible intermediate states could exist, depending upon the “lid” position, but its mobility is also influenced by other residues and the molecules present in the system (Louwrier et al. 1996). It is believed that the opening and closing mechanism is involved in lipase catalytic mechanism (Gonzalez-Navarro et al. 2001; Grochulski et al. 1994). In low micelles/interface system, it is suggested that the closed state predominates, resulting in low enzyme activity. In contrast, in water-lipid interface, the “lid” could interact with this interface undergoing a conformational change, opening and exposing the site. This phenomenon, called interfacial activation, is described by many authors as crucial for lipase activity. Also, it was shown that lipases may exist in a dynamic equilibrium between opened and closed conformations. Figure 11.8 illustrates this state of lipase, where open and close lids are represented. However, neither the “lid” nor the interfacial activation is characteristic of all lipases. Nevertheless, the literature has shown that most of the lipases undergo profound conformational changes in interfacial activation and that the “lid” moves outward from lipase, leaving the enzyme in an active form exposing the catalytic site for the protein-lipid interaction.

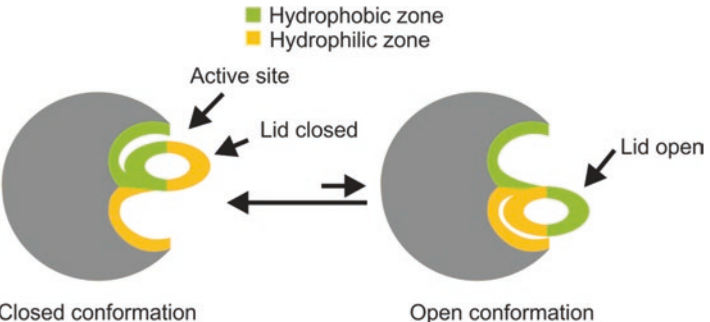

Fig. 11.8 Interfacial adsorption of lipases

11.6 Lipase Kinetics: An Actual State of Discussion

The critical micelle concentration, CMC, leads to a heterogeneous system where the enzyme acts on the interface between both polar and nonpolar substances, and the arrangement of these layers are proposed and studied in terms of lipase kinetics, from monolayers (Ivanova et al. 2002), micellar systems (Berg et al. 1991), in biphasic systems (Hermansyah et al. 2010; Tsai and Chang 1993), and the reversed micellar systems (Jurado et al. 2006; Knezevic et al. 1998; Shiomori et al. 1996; Tsai and Chiang 1991). All of them considered the existence of a crucial interface for lipase activity. One explanation to this is the interfacial activation, a phenomenon caused to the unfolding of the hydrophobic peptide loop that covers the active site of the enzyme when it is attached to the lipid-water interface, undergoing a conformational rearrangement, making the active site accessible to the substrate. As explained by Guerra et al. (2011), when the substrate concentration is lower than its CMC, the lipase barely reacts (Fig. 11.9a); however, when the water-lipid interface is created, as in high substrate concentration beyond CMC or adding a nonpolar solvent (*e.g.*, hexane or isopropanol) or even those formed by emulsification agents (*e.g.*, Triton X-100, Tween 20), the equilibrium shifts toward the active open conformation, increasing the initial velocity of the reaction (Fig. 11.9b). The nonlinear relation between substrate and lipase concentrations and the cooperative effects in the adsorption of lipases to the interface might explain the sigmoidal activity in substrate concentration/velocity profiles when no interfacial activation is occurring (Oliveira et al. 2015).

The concerning discussion is that many authors tried to describe a mathematical model for lipase activity (hydrolysis, esterification, and, more recently, transesterification), although discrepancies among results have been found. Various parameters are assumed when describing lipase kinetics leading to various models described

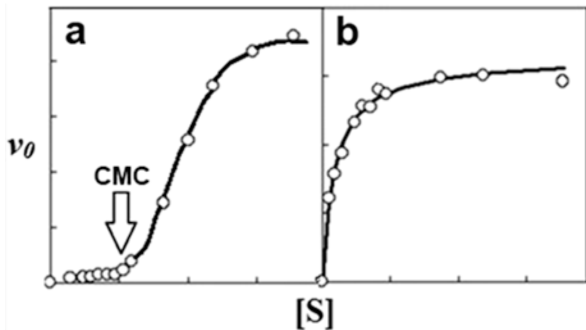

Fig. 11.9 Hypothetical representation of initial velocity (v_0) and substrate concentration ([S]) plots in the absence of interfacial activation (**a**) and in interfacial activation (**b**). In (**a**), there is a shift in lipase activity when substrate concentration reaches its CMC (indicated by an arrow), resulting in an increase of activity. In (**b**), the presence of a detergent of a nonpolar solvent triggers lipase-catalyzed reaction even at low concentrations of substrate by forming the interface necessary to enzyme activation

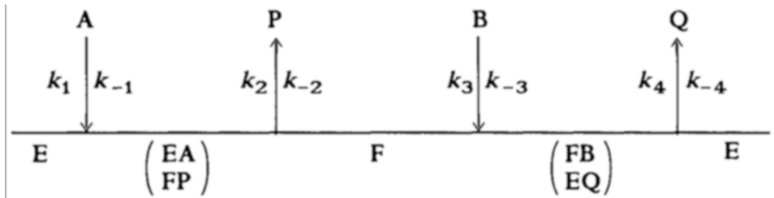

Fig. 11.10 The ping-pong bi-bi model summarized. "A" and "B" are the substrates, "P" and "Q" are the products generated after the full reaction sequence. "E" is the free enzyme, and "F" is the free enzyme modified by the first substrate. "EA" is the enzyme complex with the first substrate, "FP" is the enzyme-modified complex with the first product, "FB" is the enzyme modified complex with the second substrate, and "EQ" is the enzyme complex with the second product. k_1, k_2, k_3, $k_{4,}$ $k_{-1,}$ k_{-2}, k_{-3}, and k_{-4} are the constant rates. (Modified from Bisswanger (2002)

in literature. Firstly, it is assumed that lipase activity is not explained by Michaelis-Menten model (Al-Zuhair 2006; Hermansyah et al. 2007), which states that an enzyme catalyzes the reaction of one substrate to one product. Chulalaksananukul et al. (1990) proposed the ping-pong bi-bi mechanism to explain lipase-catalyzed esterification of oleic acid and ethanol esterification by *M. miehei* lipase (Fig. 11.10).

This model states that two substrates and two products are necessary for a complete cycle of reaction; the enzyme reacts with the first substrate forming a covalently acyl-enzyme and then releases the first product. The second substrate reacts with the acyl-enzyme complex, forming the second product (Bisswanger 2002). The ping-pong bi-bi model double reciprocal plots have parallel lines when varying the concentration of one of the substrates, while the sequential mechanism plots have lines with different slopes. By this statement, many authors are referring on ping-pong bi-bi model to explain lipase-catalyzed reactions (Bousquet-Dubouch et al. 2001; Chew et al. 2008; Hermansyah et al. 2010; Jamie et al. 2017; Janssen et al. 1999; Méndez et al. 2009; Veny et al. 2014). The substrates and products for each catalyzed reaction are described in Table 11.2.

Equation 11.1 summarizes the kinetics of lipase-catalyzed reaction by ping-pong bi-bi.

$$v = \frac{v_{\max}[A][B]}{K_{\mathrm{b}}[A] + K_{\alpha}[B] + [A][B]} \tag{11.1}$$

Since lipase reactions depend on interfacial activation, it is addressed that this particular characteristic should be included in the proposed model. Al-Zuhair et al. (2003) and Tsai and Chang (1993) proposed a hydrolysis kinetic equation which includes the interface activation previously described. The hypothesis assumes that the lipase is absorbed into the interface to yield an activated enzyme, E^*. This absorption is proportional to the free area of the interface, a, and free enzyme concentration, E. The enzyme-substrate complex, E^*S, is then formed by the interaction between the active enzyme and a substrate molecule. This leads to generate the

Table 11.2 Majority of chemical groups of substrates and products for each catalyzed reaction by lipase

Reaction	Substrate A	Product P	Substrate B	Product Q
Hydrolysis	Ester	Alcohol	Water	Fatty acid
Esterification	Fatty acid	Water	Alcohol	Ester
Transesterification	Ester	Short alcohol	Alcohol	Fatty acid short alcohol ester

product P^* and the enzyme E^* at the end of the reaction. Finally, the product P^* is desorbed from the interface to the bulk phase as final product, P.

As proposed by Tsai and Chang (1993), a quasi-linear steady state was assumed for the enzyme-substrate adsorption and desorption system which can lead to Eq. 11.2.

$$v = \frac{V_{\max}[S]}{K_e\left(\frac{k_d}{k_a a_t^2}+1\right)+[S]} \tag{11.2}$$

where $K_e = (k_{cat} + k_{-1})/k_1$ and $k^*_{cat} = k_{cat}/ C^*$, in which C^* describes the ratio of product, P^* is converted to P in the bulk phase, k_p is the constant rate of enzyme absorption to the interface, and k_d is the constant rate of desorption of enzyme from the interface.

This brings to discussion many aspects of lipase kinetics and conflicts between experimental data. Al-Zuhair et al. (2004) described that Eq. 11.2 does not fit for high enzyme concentration. Also, since the interface is crucial, the application of emulsification reagent (like those in micellar systems) or agitation during hydrolysis would trigger a huge interfacial area, and the initial velocity would be resumed as a normal Michaelis model (Al-Zuhair 2006).

In terms of inhibition, the interpretation of lipase kinetic experiments can be complicated. Even if the catalytic conversion of the reverse steps has high activation energy, the products, which might have some structural resemblance to the reagents, could inhibit the enzyme as they could compete for the binding site. Also, the products and reagents could bind to any step as shown in (Fig. 11.10), creating many possibilities of inhibition. Janssen et al. (1999) described that solvent, alcohol, and fatty acids influence the esterification kinetics of lipase from *C. rugosa*. It was shown that the last two are inhibitors of the reaction, and the solvent affects the proximity of the substrates of reaction. The same was written by Méndez et al. (2009), showing a possible *n*-propanol and fatty acid inhibition during esterification by lipase from *Rhizopus oryzae*. This effect of inhibition was also present at the hydrolysis as demonstrated by Chew et al. (2008). In their study of hydrolysis of palm olein using immobilized commercial lipase, water and fatty acids are capable of inhibiting the hydrolysis reaction. Sun et al. (2013) proposed a bi-bi model for transesterification of palm oil and dimethyl carbonate in which no inhibition effect was considered, neither from substrates nor the products, but its model differs from Veny et al. (2014) that include a methanol reversible inhibition step.

Besides the literature, lipase kinetics is not a defined concept. Many assumptions must be considered when proposing a model. The inhibition by-products and substrates, the nonspecific lipase cleavage of ester bonds of the glycerol backbone, the relationship between interfacial and bulk concentrations of the enzyme, and the agitation and interfacial area are parameters that should be included (or at least taking in account) when formulating a kinetic model, especially when applying to an industry. Tailoring reaction parameters is the key factor to debottleneck the difficulties of white biotechnology. Understanding what parameters affect the rate of enzyme catalysis and describing models capable to predict the amount of reagent, products, the rates of their depletion and generation, respectively, and also other parameters that inhibit or promote the activity of lipase will have a great impact on the final products. The kinetic study of lipase (as well as other enzymes) is a tool capable of managing the budget and profitability of the industries that utilize their chemical nature.

11.7 Lipase Purification

Eukaryotic microorganisms secrete their lipases mostly in the extracellular environment. These enzymes are recovered by filtration or centrifugation, and the supernatant is concentrated using ultrafiltration, extraction with organic solvents, or precipitation. According to Aires-Barros et al. (1994) about 80% of all purification strategies include a precipitation step, and 60% of these procedures use ammonium sulfate. In addition, acetone, ethanol, and acid are commonly used in such phases. These procedures are usually considered as preparatory for further purification. Lipase purification steps are based on the properties of the protein and their interaction with chromatographic resins, such as liquid ionic charge, molecular weight, hydrophobicity, specificity, etc., which is in accordance with other proteins. These strategies may involve one or several steps, with variable final recovery. For example, the *Beauveria bassiana* lipase A was efficiently purified on octyl-Sepharose chromatography, with a recovery and purification factor of approximately 75% and 15, respectively (Vici et al. 2015). On the other hand, the *Mortierella alliacea* lipase was purified in three steps – acetone precipitation and sequential chromatography on diethylaminoethyl (DEAE)-Sepharose and Superdex G-100 – with a recovery of 4% and purification factor of 6.2 (Jermsuntiea et al. 2011). Table 11.3 shows a summary of fungal lipase purification.

Frequently, the more steps required for purification, the lower is the protein recovery. Nevertheless, in many cases the purification in a single step does not provide a desirable purity level for the study of the enzyme. The application of an enzyme in the health/pharmaceutical area requires high degree of purity, for example. On the other hand, its application in biofuel industry does not require such refined procedure. Therefore, the purification procedure should be developed according to the final application of the enzyme, thus avoiding unnecessary expenses with the product.

Table 11.3 Summary of conventional lipase purification techniques for fungi enzymes

Fungus	Purification strategies	PF[a]	PR[b] (%)	MW[c] (kDa)	Reference
Antrodia cinnamomea	Ammonium sulfate PPT[d] and phenyl-Sepharose	17.2	33.6	60	Shu et al. (2006)
Aspergillus awamori (BTMFW032)	Ammonium sulfate PPT and DEAE cellulose	30.2	33.7	90	Basheer et al. (2011)
Aspergillus fumigatus (expressed in *Escherichia coli*)	Ni-NTA agarose	8.47	86.1	38	Shangguan et al. (2011)
Aspergillus japonicus LAB01	Ammonium sulfate PPT and Superose 12HR gel filtration	3.91	44.2	25	Souza et al. (2014)
Aspergillus niger F044	Ammonium sulfate PPT, DEAE-Sepharose Fast Flow, and Sephadex G-75	73.71	33.99	35–40	Shu et al. (2007)
A. niger F044 (expressed in *P. pastoris*)	Ni-NTA agarose and Sephadex G-75 gel filtration	–	–	35–40	Shu et al. (2009)
Aspergillus terreus NCFT 4269.10	Ammonium sulfate PPT and Sephadex G-100	2.56	8.44	46.3	Sethi et al. (2016)
Beauveria bassiana (expressed in *P. pastoris*)	IMAC – Cu^{2+} octyl-Sepharose	15.30 13.88	39.13 75.58	78	Vici et al. (2015)
Fusarium solani N4-2	Acetone PPT and Q-Sepharose	34	42	31.6	Liu et al. (2009)
Fusarium verticillioides	Lipase 1 – octyl-Sepharose Lipase 2 – octadecyl sepabeads	2.14 4.11	44.5 25.7	30.3 68.0	Facchini et al. (2018)
Mortierella alliacea	Acetone PPT, DEAE-Sepharose, and Superdex 200	6.2	4	11	Jermsuntiea et al. (2011)
Penicillium camemberti Thom PG-3	pH PPT, ethanol PPT, ammonium sulfate PPT, and DEAE cellulose	22.1	8.7	28.18	Tan et al. (2004)
Penicillium cyclopium	Ammonium sulfate PPT, Sephadex G-75, DEAE-Sephadex, and Sephadex G-75 again	590	30	40–43	Chahinian et al. (2000)
Rhizopus arrhizus	Sephadex G75-3 fractions: Lip I	1.13	1.0	80	Dobrev et al. (2011)
	Lip II	2.95	6.6	39.7	
	Lip III	0.03	2.3	6.9-	
Rhizopus chinensis	Ammonium sulfate PPT, butyl-Sepharose, and Superdex 75	138.3	0.7	33	Sun et al. (2009)
Talaromyces thermophilus	Ammonium sulfate PPT, Sephacryl S-200 gel filtration, and MonoQ FPLC	105.75	29.13	39	Romdhane et al. (2010)

[a]Purification factor; [b]Protein recovery; [c]Molecular weight; [d]Precipitation

Table 11.4 Summary of novel methods for fungal lipase purification

Fungus	Purification strategies	PF[a]	PR[b] (%)	MW[c] (kDa)	Reference
Aspergillus niger	RMS[d] (isooctane/butanol/ hexane, 75/15/10 (v/v/v)) with 0.2 M CTABg, pH 9	4.09	82.72	32	Nandini and Rastogi (2009)
A. niger	ATPS[e] (PEG 4000/Ci), pH 5.2	30.5	95.14	–	Marini et al. (2011)
Candida antarctica	ATPS (25% w/w [C8mim] Cll/30%), 25 °C, pH 7	2.6	95.9	35.3	Ventura et al. (2011)
Candida rugosa	Immunopurification with monoclonal antibodies: BF11/VNH9	–	99/92	60	Rahimi et al. (2004)

[a]Purification factor; [b]Protein recovery; [c]Molecular weight; [d] Reverse micellar system; [e] Aqueous two-phase system

Commonly, such purification strategies involve at least one hydrophobic interaction chromatography step, predominantly with octyl or phenyl resins. This artifice is based on a characteristic present in many lipases: the hydrophobic region around the catalytic site of the enzyme. Due to this characteristic, lipases tend to bind more strongly to hydrophobic resins as compared to other proteins. This binding may be so strong that it is often necessary to use detergents for the desorption of the enzyme. Although such classical methods of purification are still widely used, novel methods have been developed for the purpose of facilitating the process, enhancing recovery and purity of the enzyme. According to Tan et al. (2015), some of these new methods include reverse micellar system (RMS), immunopurification, and aqueous two-phase system (ATPS). Table 11.4 summarizes some of these procedures applied to fungal lipases.

11.8 Lipase Immobilization

The immobilization of enzymes plays an important role in biotechnology. Although it has other benefits, the main reason for immobilizing enzymes is the ease of isolating the biocatalyst from the final product and reusing it as much as possible in order to increase productivity. In the case of lipases, immobilization may help provide nonaqueous conditions which are necessary for synthesis reactions, such as esterification, interesterification, and transesterification. In addition, immobilization often improves enzyme characteristics, such as organic solvents and thermal stability. In many cases, they modulate the activity of the enzyme, modifying, for example, regio-, enantio-, and stereospecificity which are very important in the case of the lipase application in food and pharmaceutical industries. Pereira et al. (2017) demonstrated that *Hypocrea pseudokoningii* lipase had its enantioselectivity modified depending on the type of covalent immobilization used. In this case, Glyoxyl and cyanogen bromide (CNBr) derivatives preferably hydrolyzed the S-isomer of

butyryl-2-phenylacetic acid racemic mixture, while the glutaraldehyde and glutaraldehyde cross-linked derivatives preferably hydrolyzed the R-isomer.

There are different immobilization protocols. Many of them rely on the enzyme characteristics to bind themselves to resins similar to those used for enzyme purification. For lipases, some examples to be cited are the immobilization by adsorption on hydrophobic supports, such as octyl (Fig. 11.11a), or ionic, such as DEAE type

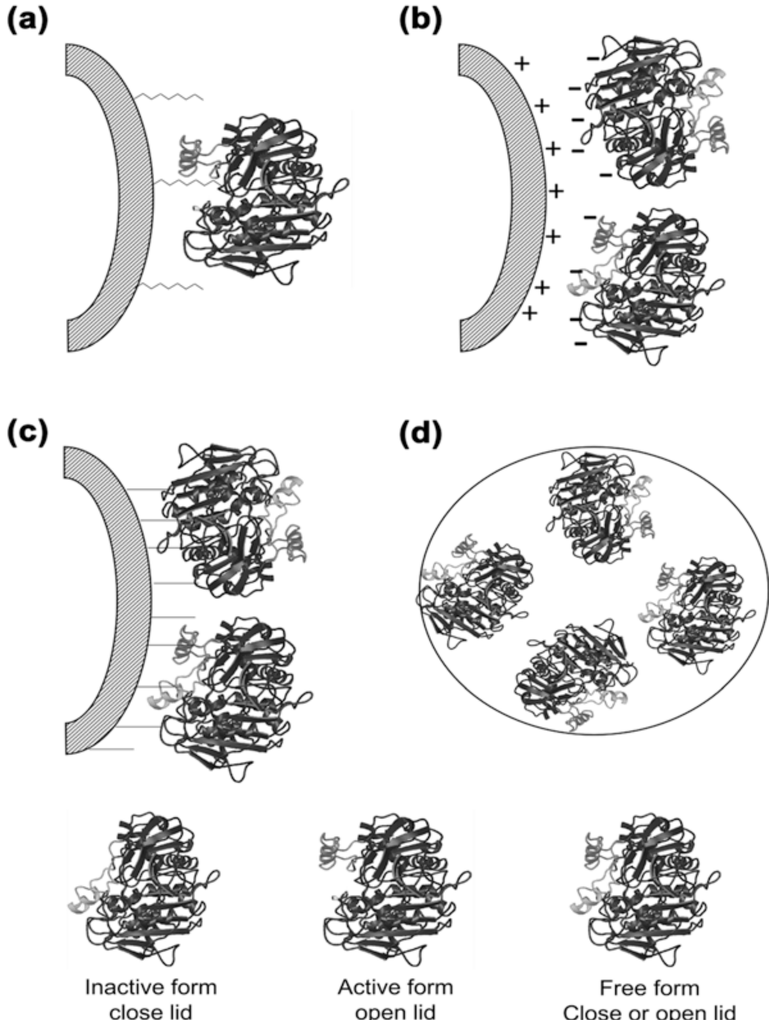

Fig. 11.11 Lipase immobilization in different types of supports. (**a**) Hydrophobic interaction, (**b**) ionic interaction, (**c**) covalent immobilization, (**d**) encapsulation. Hydrophobic immobilization usually stabilizes the active form of lipases. In other types of immobilization, the conformation can be variable, with the active site free or not

(Fig. 11.11b). Other classical forms of immobilization involve (i) covalent attachment, for example, in supports functionalized with epoxide or aldehyde groups (Fig. 11.11c); (ii) encapsulation (Fig. 11.11d) of the enzyme, in alginate beads, for example; (iii) adsorption on charged polymers; and (iv) cross-link producing aggregates of proteins or bound to a physical support, such as those obtained using glutaraldehyde.

Lipase immobilization in hydrophobic supports can stabilize the enzyme in its active form, leaving the active site more available to the substrate (Fig. 11.11a). In the case of lipases that have a "lid", as illustrated above, the hydrophobic immobilization may keep the "lid" in the open conformation. In many cases, this immobilization type promotes the enzyme hyperactivation. Manoel et al. (2015) demonstrate that lipases produced by *Thermomyces lanuginosus* and *Pseudomonas cepacia* immobilized on octyl agarose presented their open form stabilized, while the covalent preparation with CNBr-agarose maintained a closing/opening equilibrium. *Hypocrea pseudokoningii* lipase presented threefold activation when immobilized in octyl-Sepharose (Pereira et al. 2015). *Fusarium verticillioides* lipases were activated in 3.7-, 2.6-, 2.4-, and 2.6-fold when immobilized in phenyl-Toyopearl, hexyl-Toyopearl, octyl-Sepharose, and octadecyl sepabeads, respectively.

The use of heterofunctional supports for enzyme immobilization is found in literature. For lipases, normally one of the groups present in these supports is of hydrophobic interaction – to stabilize the active conformation of the enzyme – and the second group is of covalent attachment, such as glyoxyl. Rueda et al. (2015) used octyl-glyoxyl agarose support to immobilize the lipases from *C. antarctica* (form B), *Thermomyces lanuginosus* (TLL), or *M. miehei*. In this case, the immobilization occurs in two steps: (i) first the enzyme is bound to the support via hydrophobic interaction, at pH 7.0 – in this process, the protein stabilization and interfacial activation may occur – and (ii) subsequently, the pH is raised to 10 and covalent attachment to the aldehyde groups occurs. Thus, it is possible to combine two benefits: the activation of lipase in hydrophobic support and the stability and irreversibility of the covalent bond.

There are several types of support variations used for immobilization, such as agarose, chitosan, dextran, and various synthetic materials, being many of them, with the same types of binder groups. It is possible to find several papers that use nanomaterials, such as nanotubes and magnetic nanoparticles. For example, the lipase from *C. rugosa* was immobilized on magnetic nanoparticles supported ionic liquids (Jiang et al. 2009). Lipases are also found immobilized on sol-gel, such as *C. rugosa* lipase, and immobilized in hydrophobic sol-gel, with silica matrix and encapsulated in the presence of polyethylene glycol (PEG-1450) (Soares et al. 2004). Other type of lipase immobilization is the reverse micellar system. These immobilization systems can also be combined, as done by Yi et al. (2017) who developed a reverse micelle strategy for fabricating magnetic lipase-immobilized nanoparticles.

The type of support and the linker groups should be studied mainly for the application of the enzyme. For example, agarose-based supports are not ideal for nonaqueous reactions, such as transesterification. Supports with ionic groups are not recommended for applications where high ionic strength is required.

11.9 Lipases as Versatile Tools to White Biotechnology

Considering the white biotechnology, or simply industrial biotechnology, enzymes from microorganisms are industrial catalysts that produce either valuable chemicals or destroy polluting/hazardous chemicals. White biotechnology tends to have a number of advantages over traditional chemical processes, since they tend to consume fewer resources than the traditional processes used to produce industrial goods. Lipases are a good example of enzyme used in white technology. They have several biotechnological applications due to their versatile properties, and they are mainly important in biodiesel processing, pharmaceutical, food, and detergent industries. However, several other industrial sectors also contemplate the use of lipases, as will be described below (Andualema and Gessesse 2012; Guerrand 2017; Shelatkar et al. 2016; Yadav et al. 2017). A summary of the various application of lipase is illustrated in Fig. 11.12.

11.9.1 Lipases in Fat and Oleochemical Industry

The use of enzymes in the oil and fat industry has taken on special relevance despite being relatively new. Because lipases are able to catalyze many of the reactions under mild conditions and produce compounds with high purity, they are used by

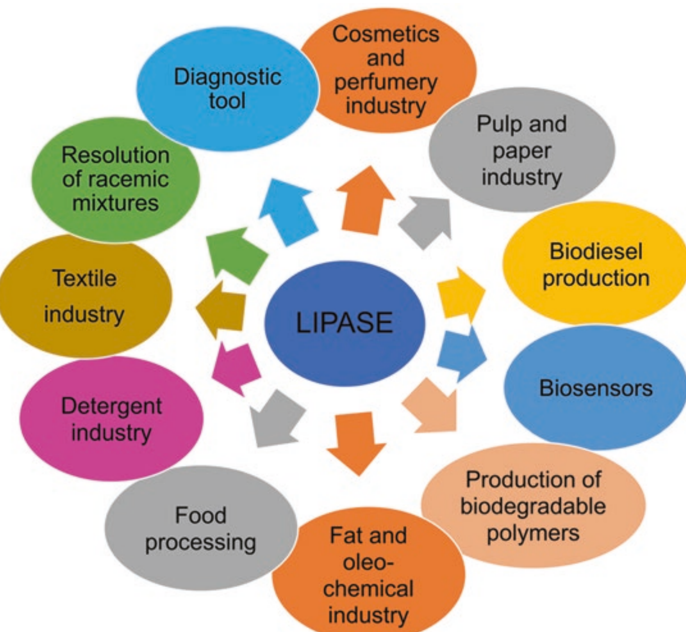

Fig. 11.12 Potential lipase applications

the industry to obtain new or modified fats and oils. In the fat and oleochemical industry, lipases perform mixed hydrolysis and synthesis reactions, allowing the production of compounds with high nutritional value and whose production by other methodologies can be very expensive for the industry, which would increase the prices of these products in the market. For example, the fat of cocoa butter needed for chocolate production is often scarce, and the price may widely fluctuate in the market. Lipase-catalyzed transesterification of cheaper oils can be used to produce cocoa butter from the middle palm fraction. In addition, lipases can be used for the production of human milk fat substitutes, polyunsaturated fatty acids (PUFAs), and biodiesel production from vegetable oils.

Lipase is used to enrich food with PUFAs from animal and plant lipids. Free PUFAs and their mono- and diglycerides are subsequently used to produce a variety of pharmaceuticals (anti-inflammatories, thrombolytics, weight loss products, and others). Because of their metabolic effects, PUFAs are increasingly used as pharmaceuticals, nutraceuticals, and food additives. Many of the PUFAs are essential for normal synthesis of lipid membranes and prostaglandins. PUFAs have beneficial effects on human health, since they are able to activate the immune system, protecting the body from numerous infectious diseases. Moreover, these essential fatty acids protect the body from important diseases like diabetes and cancer. Larsson et al. (2004) reported that people who consume these fatty acids in their diet often have less risk of developing various types of cancer such as brain and stomach. Microbial lipases are used to obtain PUFAs from animal and plant lipids such as fish oil, açai oil, and borage oil.

Free and immobilized lipases in different supports are able to catalyze the production of new oils, such as corn oil, soybean oil, peanut oil, and sesame oil. Lipases isolated from *M. miehei* were used for this purpose. Additionally, these enzymes are used for the hydrolysis of oils and fats to produce free fatty acids and glycerol, compounds which are later used in the industry for the production of soap, to generate flavor in various types of foods, and as precursors of various medicines in the pharmaceutical industry (Andualema and Gessesse 2012). Annually 2 million tons per year of fats and oils are used in high energy consumption processes such as hydrolysis, glycerolysis, and alcoholysis. The conditions for the separation of the grease from the vapor and conventional glycerolysis of the oils involve high temperatures of 240–260 °C and high pressures. In addition, because of the chemical structure of highly unsaturated fatty acids, which are not resistant to processes that occur at high temperatures, the conventional chemical methods used in the industry for carrying out processes requiring high energy consumption are being replaced by methods using enzymes (Aravindan et al. 2007).

11.9.2 Production of Biodegradable Polymers

The production of polymeric materials through the use of enzymes is an area of emerging research with a great scientific and technological importance, besides the favorable impacts for the environment. The use of lipases for the polymerization of

biodegradable compounds can offer many advantages, being more efficient than the traditional chemical and physical techniques used to obtain polymerized materials, as well as the development of new products, hitherto not accessible using traditional chemical approaches. Lipases are able to produce 1-butyl oleate by direct esterification of butanol and oleic acid. This compound reduces the viscosity of biodiesel, allowing a better use of it at low temperatures. Trimethylolpropane esters were also similarly synthesized as lubricants. The synthesis of esters and transesterification reactions in organic solvent systems is possible through the use of lipases, allowing the synthesis catalyzed by enzymes of biodegradable polyesters. Lipases may also be employed for the biocatalysis of aromatic polyesters, compounds that exhibit stability at high temperatures and extreme chemical conditions.

11.9.3 Use of Lipase in Textile Industry

Lipases at present are widely used in the leather industry, since they are able to transform the natural fat present in the skins into free fatty acids and triacylglycerol. This process facilitates the subsequent use of hydrophilic chemical compounds, allowing the tissues to become softer, without blemishes, and with a good smell. Lipases and alpha amylases have been used for the desizing of denim and other cotton fabrics. Polyester is one of the most valued fabrics in the textile industry, since it has properties such as high resistance to stretch, stains, and abrasion, it is a soft fabric, and it can be machine washed and has characteristics that prevents the development of wrinkles. Due to these advantages, the polyester fiber enzymatic treatment (lipases) allows it to increase the ability of this fabric to absorb dyes, cationic chemical compounds that aid in tissue preservation, antimicrobial and antistatic compounds, as well as chemical formulations that enable better finishing of fabrics. The enzymatically treated polyester can be used for the manufacture of yarns, fabrics, and rugs, among other consumer items.

In a study conducted using 84 sheepskins from Western Turkey, it was found that the use of commercial formulations containing lipases for the treatment of these tissues resulted in a considerable decrease in the presence of fat in the tissues. This effect, according to other studies, may be enhanced by increasing the concentration of lipases present in the commercial formulations and the time of tissue exposure to these enzymes. The skins obtained after the enzymatic treatment have favorable characteristics that allow their use for the textile industry for the manufacture of wallets and coats, among other items (Afsar and Cetinkaya 2008).

11.9.4 Lipases in Detergent Industry

The main use of lipases is in the detergent industry. The majority of commercial detergent formulations on the market have lipases in combination with other enzymes. The investment of industries in the search for lipases that can be used in

commercial detergents has considerably grown, because enzymes help in reducing the amounts of detergent in the environment as they minimize the presence of less desirable compounds in their formulation. Also, detergents which contain lipases are biodegradable, do not cause harm on human health, have no negative impact on sewage treatment, and do not affect the environment and aquatic life. Lipases used for detergent formulations can be isolated from microorganisms such as *Pseudomonas* and immobilized in numerous surfaces (Patent # 6,265,191, issued July 24, 2001). This immobilization of lipase facilitates better removal of fat stains by forming a complex with the tissue. The adsorbed lipase has greater stability to surfactant denaturation and heat deactivation, retains substantial activity after drying the tissue at an elevated temperature, and maintains activity during tissue storage or wear. The redeposition of oil by-products in the tissue is delayed by the presence of lipases in the detergents.

At present numerous investigations are being carried out for the use of other enzymes, besides the lipase in the commercial formulations of detergents. Additionally, interest to search for lipases that are capable of acting under alkaline conditions removing fat present in the tissues has been enhanced for many years. Another interesting aspect is that lipases maintain their activity in the presence of proteases and activated whitening systems, allowing the droplets of fat to be converted into chemical compounds with more hydrophilic characteristics, readily allowing their removal from tissue compared to fat without enzymatic treatment (Fujii et al. 1986). It is estimated that every year, about 1000 tons of lipases are added to approximately 13 billion tons of detergents.

Detergents containing lipases in combination with oxidoreductases can be employed in cleaning contact lens, removing grease in the sewage system of restaurants and industries, and washing of fabrics in the textile industry among other utilities. The use of lipase increases the detergency and decreases the amount of surfactant present in the detergent (Verma et al. 2012). During the years 1980–1990, many different lipases present in detergents were isolated from fungi such as *Humicola lanuginosa*. The amount of lipase produced by fungi is frequently inefficient for the industry; due to this restriction cloning strategies of genes encoding lipase have been developed, and then, these genes were inserted into other fungi such as *Aspergillus oryzae*, a filamentous fungus, used in Japanese cuisine for the fermentation of soya beans.

11.9.5 Lipases in Food Processing, Flavor Development, and Improving Quality

The microbial lipases, due to their regio- and enantioselectivity characteristics, are being widely used in the food industry for the processing and modification of oils and fats. The use of lipases for the modification and structuring of vegetable fats with triacylglycerides or free fatty acids, nutritionally important, has shown special relevance since the enzymatic modification happens in specific regions of

the macromolecules and under soft reaction conditions. Another very interesting use of lipases in the food industry is related to the synthesis of esters of short-chain fatty acids and alcohols, which are compounds responsible for the modification of flavor and fragrance of food. Hexyl acetate, a fruity odorous short-chain ester, is a widely used compound in the food industry. The hexyl butyrate synthesized by immobilized lipase (Lipozyme IM-77) from *M. miehei* was used as aroma and fragrance in the food, beverage, and pharmaceutical industries. Gram-negative bacteria, such as *Pseudomonas* species, represent a problem in the deterioration of meat, mainly by the production of enzymes such as proteases and lipases, whose enzymes catalyze the formation of compounds responsible for the bad smell of the meat.

Lipases are used in a procedure called biolipolysis, which allows the removal of fats from the meat during processing and promoting the production of lean meat. This technique has been used mainly for fish meat. Lipases also play an important role in the fermentative steps of sausage manufacture and to determine changes in long-chain fatty acid released during ripening. Lipases of different microbial origin have been used for refining rice flavor, modifying soybean milk and accelerating the fermentation, and improving the aroma of apple wine. Research in recent years has received much attention for using microbial lipases to produce omega-3 (ω3-PUFA) concentrates by hydrolysis of marine oils. The fatty acid selectivity of a lipase for eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) allows separation and concentration of these fatty acids from others in the remaining portion of marine oils. In addition, lipases have been frequently used to discriminate between EPA and DHA in concentrates containing both of these fatty acids, thus providing the possibility of producing ω3-PUFA concentrates with dominance of either EPA or DHA (Kumar et al. 2017; Shahidi and Wanasundara 1998).

Eggs are an important component of numerous foods marketed by the industry such as emulsions and mayonnaise. The emulsifying power of the egg depends on the composition of its lipids; the conversion of the phospholipids present in the egg by lipases into lysophospholipids notably increases the stability of the emulsion. This processing allows a considerable reduction in the use of egg yolks in dressings and processed foods. Lipases used for egg processing may be isolated from the porcine pancreas (e.g., Lipomod 699, Biocatalysts, UK) or from microorganisms (e.g., Maxapal A2, DSM, NL). Lipases cloned and expressed in the *Aspergillus niger* fungus (such as DSM Maxapal A2) also achieve high conversion yields, but some industry players are not in favor of using enzymes produced by such modified microorganisms, because of ethical problems (Guerrand 2017). Table 11.5 lists some uses of lipase in the food industry.

11.9.6 Resolution of Racemic Mixtures

Lipases are highly specific to the substrate that is hydrolyzed, a feature that allows lipases to be used for the resolution of racemic mixtures and for the synthesis of chiral compounds present in pharmaceuticals, agrochemicals, and pesticides. The

Table 11.5 Lipase applications in the food industry

Food industry	Action	Product of application
Dairy foods	Hydrolysis of milk fat, cheese ripening, modification of butter fat	Development of flavoring agents in milk, cheese, and butter
Bakery foods	Flavor improvement	Shelf life propagation
Beverages	Improved aroma	Alcoholic beverages, *e.g.*, sake, wine
Food sauce	Quality improvement	Mayonnaise, condiment, and whipping cream
Health foods	Transesterification	Health foods
Meat and fish	Flavor development	Meat and fish product, fat removal
Fats and oils	Transesterification, hydrolysis	Cocoa butter, margarine, fatty acids, glycerol, mono- and diglycerides

formation of individual enantiomers in the pharmaceutical and the agroindustrial industries, which are precursors of some drugs, medicines, or chemical products with chiral properties and are obtained through the use of enzymes coming from microorganisms and fungi, has taken great relevance. Baclofen is chemically (RS)-beta-(aminomethyl)4-chlorobenzene propanoic acid. This compound is obtained by the kinetic resolution of racemic flurbiprofen by the method of enantioselective esterification with alcohols through the use of an isolated lipase from *C. antarctica* (Zhang et al. 2005). This lipase showed excellent results in relation to chemo-, regio-, and enantioselectivity, leading to high rates of baclofen formation. The latter compound is used in pain therapy and as a muscle relaxant (Muralidhar et al. 2001).

The synthesis of (−)-15-deoxyspergualin 23, an immunosuppressive agent and antitumor antibiotic, is obtained by lipase-catalyzed stereoselective acetylation of racemic7-[N,N´-bis-(benzyloxy-carbonyl)N-(guanidinoheptanoyl)]-alphahydroxy glycine 24 to corresponding S-(−)-acetate 25. The last compound is a key intermediate for total formation of (−)-15-deoxyspergualin 23 (Patel 2000) (Fig. 11.13). Lipases isolated from *Geotrichum candidum* and *C. antarctica*, respectively, were used for the preparation of chiral intermediates through biocatalytic processes. These chemical intermediates were subsequently used for the total synthesis of pharmaceutical compounds related to the elimination of bad cholesterol and for the treatment of the Alzheimer's disease.

The selective esterification of the S-isomers with butanol through the use of a porcine pancreatic lipase allows the resolution of 2-halopropionic acids, primary constituents for the synthesis of the herbicide phenoxypropionate. The preparation of enantiomerically pure herbicides and nonsteroidal anti-inflammatory drugs (naproxen, ibuprofen) can be produced directly from the R- and S-isomers of the alpha (* sub)-phenoxypropionic acids, respectively. Several companies around the world have successfully developed these chemical reactions. Commercial lipases are also used in the resolution of racemic mixtures in the hydrolysis of epoxy

Fig. 11.13 Enantioselective enzymatic acetylation of racemic alcohol 24 to corresponding S-(−)-acetate 25 and unreacted R-(+)-alcohol 24 by lipase from a *Pseudomonas* sp. (lipase AK). Structure of 15-deoxyspergualin (antitumor antibiotic and immunosuppressive agent)

alcohol esters. Very attractive intermediates for the preparation of optically active beta-blockers can be obtained from the highly enantioselective hydrolysis of (R, S)-glycidyl butyrate by lipase. Many companies are investing in the research related to the regioselective modifications of compounds polyfunctional with the application of lipases. The production of castanospermine, a drug with a high potential in the treatment of AIDS, was possible due to the regioselective modification mechanism performed by lipase (Patel 2000). The use of lipases for the synthesis of organic compounds has many advantages such as high versatility of the enzyme over a wide range of substrates which hydrolyze high rates of product of interest formation under conditions of soft reaction with chemo-, regio-, and enantioselectivity. This characteristic allows the correct separation of the enantiomeric molecule that is bioactive from that enantiomeric molecule that does not present biological activity.

11.9.7 Diagnostic Tool

Lipases are widely used in the medical profession because the levels of these enzymes in the blood are useful tools as markers for some diseases and infections and may still be drug targets in the treatment of other diseases. In the medical sector, lipases are used for the determination of serum triglyceride (TAGs) levels from the conversion of TAGs to free fatty acids and glycerol, the latter compound can be determined by enzyme-linked colorimetric reactions. In other cases, lipase can be used as a diagnostic tool, since high levels of this enzyme in the blood may be related to the development of acute pancreatitis and pancreatic lesion. The medical laboratory combines the levels of this enzyme in the blood with other medical tests

such as serum ultrasonography with trypsin, computed tomography, and endoscopic retrograde cholangiopancreatography to perform a more accurate diagnosis of acute pancreatitis.

Despite of the levels of seric lipase being used as a marker of pancreatic injury, the medical clinic shows that it is not a marker with high reliability, since other types of cells in the body are capable of producing lipases under physiological stress or under special conditions for our body, for example, the realization of an intense and prolonged physical exercise in time, the adipocytes, cells present in the adipose tissue, are able to produce this enzyme. Taking into account the above observation, the researchers developed a test to measure canine pancreatic lipase, showing the levels of this enzyme decreased in dogs with exocrine pancreatic insufficiency (Mix and Jones 2006).

One of the factors that promote the virulence of fungus *Candida albicans* is the production of a 70 kDa molecular weight lipase, which has the ability to interact with two mammalian cell subtypes: macrophages, the crucial immune cells involved in fungal control, and hepatocytes, the example of parenchymal cells compromised during fungal dissemination. The interaction of lipase/macrophages or hepatocytes induces directly cytotoxicity and promotes the depositions of lipid droplet in the cytoplasm of macrophages and hepatocytes (Paraje et al. 2008).

P. aeruginosa is an opportunistic pathogen and nowadays is one of the most common hospital infections in patients. Its ability to synthesize and secrete different virulence factors are considered as biological properties that contribute to the pathogenicity of *P. aeruginosa*. Among the virulence factors are many enzymes, including lipases. A study that analyzed 103 samples of *P. aeruginosa* strains isolated from cancer patients showed that these strains showed high levels of lipase that correlated with a higher pathogenicity of *P. aeruginosa* strains and a worse prognosis of the cancer in the patients analyzed in the study (Lott and Lu 1991; Majtan et al. 2002).

Lipase produced by *Propionibacterium acnes* can be a factor that helps in the production of butyric acid and free fatty acids in skin diseases such as axillary seborrheic dermatitis and acne vulgaris, respectively. Several studies have shown an association between increased lipase concentrations produced by *P. acnes* and the development of infectious skin diseases. Lipase of pathogenic bacteria such as *P. acnes*, *Corynebacterium acnes*, and *Staphylococcus aureus* has also been found to have influence on skin rash in acne patients.

11.9.8 Bakery Products, Confectionery, and Cheese Flavoring

Lipases are often used in the dairy industry. Lipases are found naturally in raw milk; however, the content of lipase in raw milk may vary according to the type of animal and diet it receives; many commercial lipases are employed in the processing of

milk. Lipases can be used mainly for the processing of fat present in milk and for enhancing the flavor and maturity of the cheeses. When the fats present in the milk are broken, the lipases can produce free fatty acids of different carbon chain lengths; these differences can generate different flavors in cheeses, e.g., short-chain fatty acids (mainly C4 and C6) leads to the development of a strong and acidic flavor, while the release of medium chain fatty acids (C12, C14) tends to impart a sweet taste in the products. In addition, free fatty acids, when processed by the microbiota present in the cheese, participate in the formation of other flavoring ingredients such as acetoacetate, beta-keto acids, methyl ketones, flavor esters, and lactones.

There is a wide range of commercial lipases that are used by the dairy industry to enhance and modify the taste of cheeses. These lipases are highly appreciated mainly by the Italian gastronomy that uses a great variety of cheeses in the confection of many typical foods of the country. Example of these lipases are *M. miehei* (Piccnate, Gist-Brocades; Palatase M, Novo Nordisk), *A. niger* and *A. oryzae* (Palatase A, Novo Nordisk; Lipase AP, Amano; Flavor AGE, Chr. Hansen), and several others (Table 11.6). Another interesting application of lipases is in the preparation of the so-called enzyme-modified cheeses (EMC). These cheeses are obtained by incubating them in the presence of specific enzyme and at elevated temperature to produce a concentrated taste which can be used later as an ingredient in food products such as sauces, biscuits, soups, and snacks. The advantages of EMC cheeses in relation to other types of cheese are in the concentration of the flavor, allowing the need of a small amount of the product to obtain the same flavor intensity; in addition these cheeses have a half useful life extended, and the costs of production are decreased (Jooyandeh et al. 2009).

Lipases have also been used for processing the coffee, allowing a greater whiteness of the coffee, enhancing the flavor, and giving it a creamier texture. Additionally in the bakeries, numerous lipases, for example, lipases from *A. niger*, *R. oryzae*, and *C. cylindracea*, are used in baking products. Lipases help to increase the shelf life of the bread and favor the growth of masses and volume of the bread, besides increasing the quality of the bread structure. Numerous industries have been dedicated to the production of commercial enzymes, among them lipases, for their use in the manufacture of various bakery products.

Table 11.6 Examples of lipase in cheese production]

Cheese type	Lipase source
Romano	*Kid/lamb pre-gastric*
Domiati	*Mucor miehei*
Camembert	*Penicillium camemberti*
Mozzarella	*Calf/kid pre-gastric*
Fontina	*Mucor miehei*
Roquefort	*Penicillium roqueforti*
Cheddar	*Aspergillus oryzae/Aspergillus niger*

11.9.9 Lipases in Cosmetics and Perfumery

Cosmetics in general are defined as a class of products used for personal care. Cosmetics market has grown at a great speed over time, requiring ways to obtain better quality products and to develop more advanced technologies for its manufacture. Lipases are widely used in the manufacture and confection of cosmetics and perfumes. In the production of cosmetics, they can function both as active ingredients in cosmetic formulations and as biocatalysts in the synthesis of specific cosmetic chemicals. The aroma and fragrance are one of the main and desirable characteristics in cosmetic products. They are more frequently obtained through the synthesis and resolution of racemic mixtures and the formation of highly pure chiral compounds. Lipases have been used to obtain 1-(−)-menthol from the racemic resolution of methyl benzoate. Menthol is considered to be one of the most important odor agents for many applications and is the main compound of natural mint oil which gives many cosmetic products the typical cooling/refreshing effect.

There has been an increase in the production of modified fatty acids, as well as esters of fatty acids and fatty alcohols, which are compounds responsible for the fragrance in many creams used for the treatment of the skin and bronzer effect. These products, used in many industries, have been obtained from different types of lipases, produced by organisms such as *M. miehei* lipase and *C. cylindracea* lipase. The use of these enzymes by industries offers numerous advantages such as obtaining better quality products and at lower costs in the processing as well as the final products. Unichem International (Spain) launched the production of isopropyl myristate, isopropyl palmitate, and 2-ethylhexyl palmitate to be used as an emollient in personal care products; the production of these compounds was carried out through the use of an immobilized lipase, allowing the formatting of final products with a higher purity.

Lipases which are intended to act as an active ingredient in a cosmetic product are often encapsulated in liposomes or nanoparticles, because this procedure promotes the maintenance of lipase activity for an extended period of time and favors an efficient absorption of the cosmetic product through the skin. Recently, hollow spheres of inorganic silica have also been reported as promising materials for lipase encapsulation because of their high strength, low cost, and the pleasing hand feel of the resulting cosmetic formulations (Ansorge-Schumacher and Thum 2013).

Vitamin A (retinol) and vitamin C (ascorbic acid) are ingredients of great value for the preparation of cosmetics, which are mainly intended for skin care, because these compounds protect the skin from the action of free radicals from ultraviolet light. However, the direct application of these compounds to the skin is impossible because of the reduction in stability, irritating effects on the skin, and low solubility in water. The above drawbacks faced by industries would be facilitated by the development of synthetic products analogous to these vitamins through the use of immobilized lipases. Lipases have also been used in hair-waving preparation and used as a component of topical antiobese creams.

11.9.10 Lipase Applications in Medical and Pharmaceutical Sectors

The application of lipases is important in pharmaceuticals in the reaction of trans-esterification and hydrolysis. Lipases used in the medical and pharmaceutical sectors can be isolated from various sources such as bacteria, yeasts, fungi, and some protozoa. *C. rugosa* lipases immobilized on nylon supports and in the presence of organic solvents are capable of synthesizing lovastatin, a drug widely used in the treatment of serum cholesterol reduction. A study was developed with lipases produced by *Serratia marcescens*, which are capable of producing chiral 3-phenylglycidic acid, through an enantioselective hydrolysis. This is an intermediate compound in the synthesis of diltiazem hydrochloride, a drug used in many countries as a coronary vasodilator (Sharma and Kanwar 2014).

Another interesting aspect is that lipases, due to their ability to emulsify fats, can be used in digestive disorders that together with other enzymes, as proteases, can help in the digestion. Lipases isolated from plants have been used for this purpose, favoring the production of supplements such as Similase; All-Vita NorthWest, Vitaline® Herbal Form, manufactured by Health Care Professionals, Oregon, United States of America (USA) (Hasan et al. 2006). Another likely use of lipases is in the treatment of cancer and inflammatory diseases such as obesity and diabetes. These enzymes are capable of inducing the production of cytokines such as tumor necrosis factor and interleukin 17, substances related to fighting tumors and reducing the inflammation observed in the body. Formulations of many creams for the treatment of skin infections in the clinic contain lipases combined with other enzymes such as collagenases. Lipases help maintain the structure of the skin and protect it from damages caused by the environment.

11.9.11 Lipase Application in the Pulp and Paper Industry

Lipases are widely used in the paper industry, for the reduction of hydrophobic and sticky components present in wood, such as triglycerides and waxes. Lipases are capable of hydrolyzing triglycerides into free fatty acids and glycerol, which are substances with more hydrophilic characteristics and less sticky. This procedure favors the reduction and/or elimination of serious problems that the paper industry may have if these hydrophobic components were not enzymatically processed, for example, deposition of viscous substances in papermaking machines, which would break increasing the costs of production, as well as the appearance of stains and holes in the final paper. This method of wood pretreatment for papermaking has been used by many industries around the world, among them Nippon Paper Industries, in Japan, has developed a methodology that uses an isolated *C. rugosa* lipase for the hydrolysis of more than 90% of triglycerides present in wood.

The use of lipase for papermaking, in spite of being a relatively old methodology, brings many advantages to the industry such as an increase in paper whiteness, increase in cellulose pulping, reduction of chemical compounds for paper treatment, reduction in the amount of harmful substances discharged into rivers by these industries, increase in the machines' life span, and a reduction in the costs of production. The use of *Pseudomonas* lipases (KWI-56) in the paper industry resulted in an increase in paper bleaching and in a decrease of residual ink stains (Demuner et al. 2011).

11.9.12 Lipases as Biosensors

In recent years the use of immobilized lipases as biosensors has proven to be an efficient and fast method. In the pharmaceutical and food industry, the determination of triglycerides present in a sample of human fluids or food has been possible through the use of lipases. These enzymes convert the triglycerides into glycerol; the latter compound can be quantified through the use of chemical or enzymatic colorimetric methods. Other industries have used lipases as sensors to determine the presence of organophosphorus pesticides in various types of food. Another type of newly developed sensor of biodegradable polymers is the degradation by lipases and other enzymes; this process can be applied for the development of immunosensors based on disposable enzymes. Enzyme-labeled probes have been an attractive alternative as sensors for the determination of nucleic acids and other biomolecules. The use of lipases for this purpose has an efficient result, since the use of these enzymes allows the elimination of radioactive chemical compounds harmful to human health (Sumner et al. 2001).

11.9.13 Lipases in Tea Processing

During the manufacture of tea leaves, the treatment to which they are subjected plays an essential role in the final quality of the product. Lipases have shown great efficiency for the whitening of these beverages and for the development of aroma and flavor due to the production of compounds like fatty acids that enhance the flavor of the tea and increases new flavors. Lipase produced by *M. miehei* was used to reduce the content of total lipids in tea and, besides that, increases the amount of polyunsaturated fatty acids present in this beverage.

11.9.14 Degreasing of Leather

In the processing of animal hides, an essential step is the elimination of fat present in these tissues. The conventional methods used by the industries are based on mixtures of organic solvents and surfactants, but this method promotes the

production of volatile compounds that favor environmental contamination. Another important aspect is that the skins treated with these chemical compounds result in little homogeneity and have a reduced lifespan. An alternative method for the treatment of skins and hides of animals currently used by industries are lipases, since these enzymes allow the conversion of triglycerides into free fatty acids and glycerol. Lipases are highly valued by the leather industries because lipases can act in a wide pH range, allowing them to be used for the degreasing of tissues with different degrees of fat, and they also reduce the use of surfactants and other chemicals.

As lipases are able to act in the presence of low amounts of water, they allow the improvement of water-resistant leather production. The manufacturers of this type of fabric, much used for the confection of cars in the automobile industry, believe that the use of lipases helps to obtain cleaner leathers as well as reduces "fogging." Another interesting aspect is the use of lipases in combination with other enzymes such as proteases for the treatment of skins with high contents of hair and/or fat. Proteases facilitate the efficient removal of hair and allow the tissue to open, which increases lipase access to the skin and the emulsification of fats present in tissues. The result of this procedure is the production of cleaner and more resistant fabrics. *Rhizopus nodosus* lipase was used for the degreasing of suede clothing leathers from wooled sheep skins (Souza and Gutterres 2012).

11.9.15 Waste/Effluent/Sewage Treatment

The waters from meat industries, mainly pork and cattle, drained to the environment, accumulate great fat content, an aspect that is harmful to aquatic life and the maintenance of water quality of rivers and lakes. The enzymatic treatment of these waters, mainly with lipases, helps eliminating the fat layer present in the water, allowing the passage of oxygen necessary for the sustainability of the biomass. Lipases from different origins have been used for the cleaning of sewage as, for example, the lipase of *C. rugosa.*

Immobilized lipases are also used in the leather processing industries, for the treatment of water with high content of lipids. These enzymes show good degradation yield of these lipids and also increase the purity of water. Another interesting aspect is that lipases, depending on the origin of the fat present in the different types of meat, may present different degrees of fat removal in the waters of restaurants and industries. Studies have shown that pancreatic lipases of animal origin degrade fat from beef more easily compared to pork, probably due to the lipid structure present in these meats. This idea favors the research studies related to the mixtures of lipases that can be used for the elimination of fats according to each type of meat (Parmar et al. 2001).

11.9.16 Lipase in Biodiesel Production

Nowadays, the search for new methodologies for the production of biodiesel on an industrial scale has gained special relevance. The use of biodiesel as an alternative to fossil fuel presents great advantages, since the problems caused by their extinction together with the increase in the emission of toxic gases in the environment can be solved with the production of biodiesel. Biodiesel is defined as a mixture of monoalkyl esters and is considered a carbon-neutral fuel, since the carbon initially fixed from the atmosphere for the production of this fuel is later returned, closing a cycle (Fig. 11.14). Biodiesel can be obtained from different sources such as vegetable oils (soybean oil, jatropha oil, rapeseed oil, palm oil, sunflower oil, corn oil, peanut oil, canola oil, and cottonseed), animal fat (bovine tallow, lard), used cooking oil, grease (trap grease, float grease), and algae.

The esters obtained from the degradation of vegetable fats together with a mixture in suitable proportions with conventional diesel fuels can, in the short terms, be used in diesel engines. Although these mixtures are simple, the use of esters derived from vegetable oils in diesel engines becomes unviable with time. The esters derived from vegetable fats have high viscosity and can favor corrosion of the engines and the formation of free fatty acids resulting in the formation of gum by oxidation and polymerization as well as deposition of carbon. Due to previous limitations mentioned, vegetable oils can be processed by three main methodologies, pyrolysis,

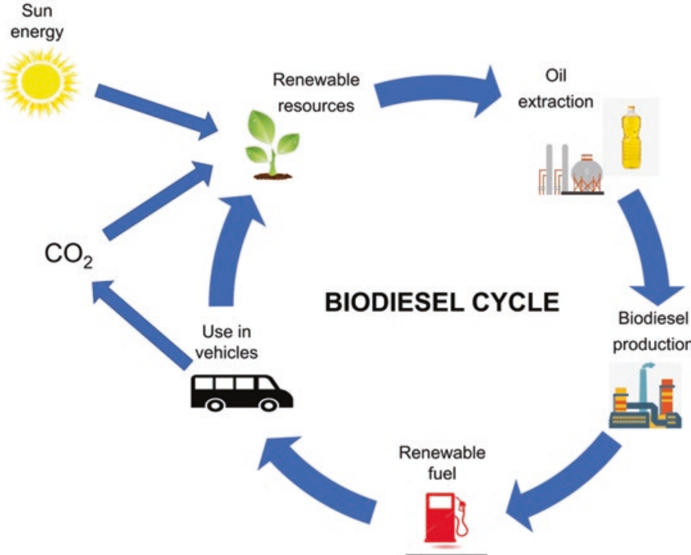

Fig. 11.14 Biodiesel cycle

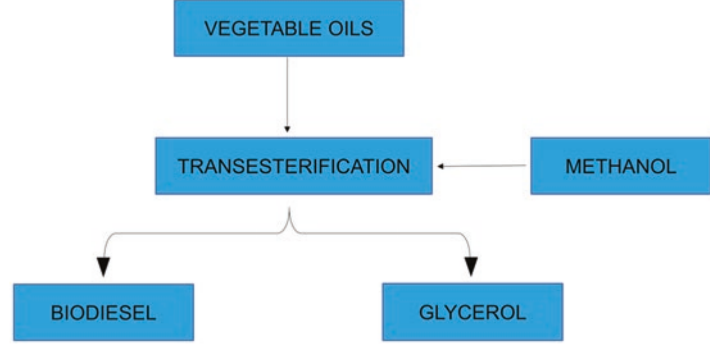

Fig. 11.15 The sequence of biodiesel production by transesterification

microemulsification, and transesterification, with the purpose of acquiring the properties of viscosity and volatility similar to fuels that make their use in diesel engines safer and longer.

Pyrolysis and microemulsification are methodologies used by industries for the production of biodiesel. However, these methodologies present some disadvantages such as the lack of some desirable characteristics in the biodiesel produced and high cost of the equipment used for its production. The use of catalysts of different nature (alkaline, acid, biocatalyst, heterogeneous catalyst, or alcohols in their supercritical state) favors an efficient transformation by transesterification of vegetable oils into biodiesel. This methodology has gained great popularity, since the alcoholysis of triglyceride esters, resulting in a mixture of monoalkyl esters and glycerol (Fig. 11.15), favors the production of biodiesel with characteristics very similar to the traditional fuels.

During the process that uses the alkaline catalyst such as sodium hydroxide (NaOH) or potassium hydroxide (KOH), the alkoxy is first formed from the mixture of the alkaline catalyst with methanol or ethanol. Subsequently the alkoxy can react with any vegetable oil to produce biodiesel and glycerol, which is deposited in the bottom of the container because it is denser, allowing the decanting of the biodiesel. Although this methodology is more efficient, since high product rates of formation are observed even at low temperatures, contamination with water or free fatty acids and the formation of soap can hinder the separation of biodiesel from the rest of the reaction components. A second strategy also used for the biodiesel production is a modification of the first strategy presented. It uses an acid catalyst in substitution to the base. Any mineral acid can be used, although the most common ones are sulfuric acid and sulfonic acid. This strategy allows the total removal of glycerol, reducing the viscosity of the biodiesel produced, a characteristic that resembles traditional fossil fuels. Although the production of biodiesel with acid catalysts is a strategy employed by the industries, it is not very efficient because these substances can damage the machines and the rates of biodiesel formation are low.

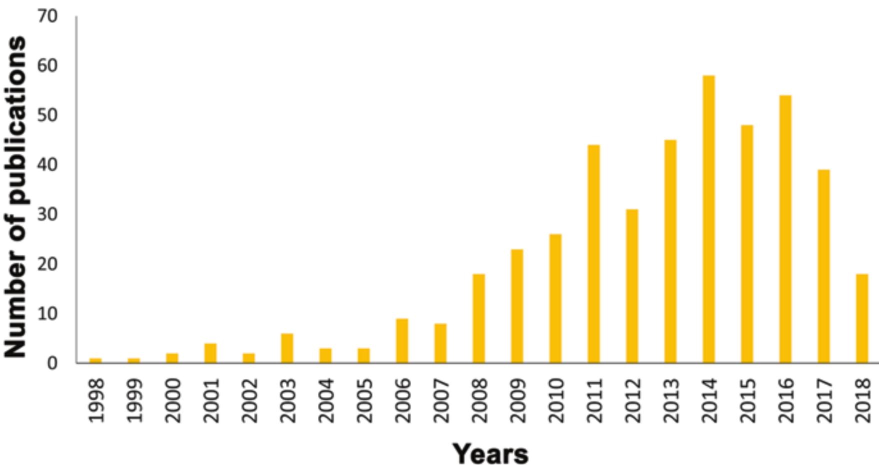

Fig. 11.16 Number of publications found in the years 1998–2018 at NCBI database using the terms “Lipase and biodiesel”

Actually, the use of immobilized lipases in different supports to catalyze the transesterification process in order to produce biodiesel has shown to be efficient. Immobilized lipases offer several advantages over the traditional methods of biodiesel production (alkaline or acidic transesterification), since it allows their reuse, and can be used without the subsequent separation of the enzyme from the products formed, and the reactions occur at low temperatures (50 °C). However, as enzymes are expensive products, it is difficult to use them on an industrial scale, and they may also be inhibited by the product, a drawback observed when methanol is used. In recent years the number of scientific publications related to lipase and its use in the biodiesel production has considerably increased (Fig. 11.16).

The method of transesterification using an alkaline catalyst has been the only method used on an industrial scale for the production of biodiesel. This method is more efficient in the formation of the product with a reasonable cost of production compared to the rest of the transesterification methodologies used with the same purpose. Despite the advantages described above, basic transesterification presents serious problems for industries such as the subsequent separation of the catalyst and methanol that did not react in the process, from the final product formed. Removal of the catalyst takes several washing steps, which can lead to difficulties in the process, and, in addition to that, the biodiesel produced must undergo successive removal of impurities to achieve the quality desired by the industry. Figures 11.17 and 11.18 compare the difference in downstream operation required for alkali and enzymatic production.

The production of biodiesel through the use of biocatalysts eliminates the limitations observed in the methodology of alkaline transesterification; since the products obtained have a higher degree of purity, fewer processes are needed to obtain the final product, and the separation of the residual reagents can be easily performed

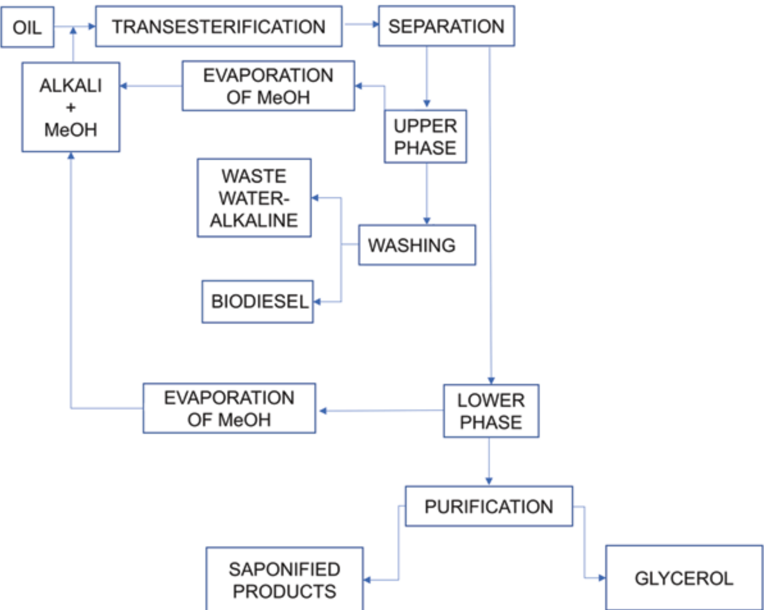

Fig. 11.17 Production of biodiesel by alkali process. MeOH - Methanol

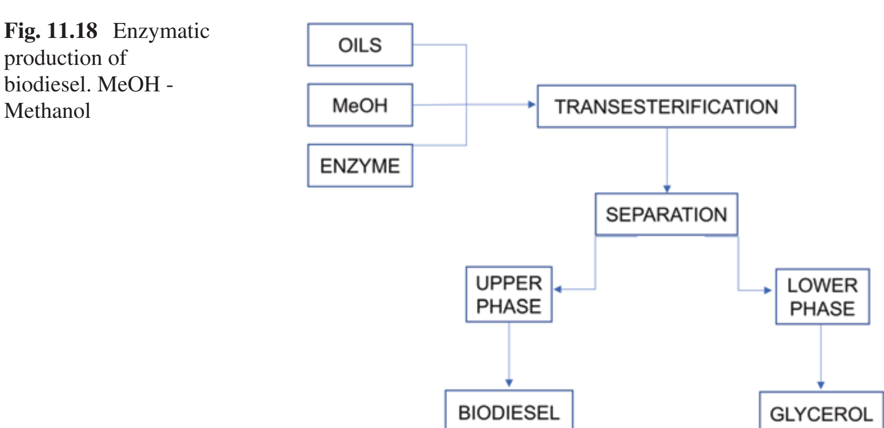

Fig. 11.18 Enzymatic production of biodiesel. MeOH - Methanol

once the biocatalyst is immobilized. Despite all the advantages shown, the process of producing biodiesel using enzymes as catalysts has not yet been implemented on an industrial scale due mainly to the high costs that the methodology demands. Several substrates are being used for the production of biodiesel from the use of the enzymatic method as, for example, methanol, ethanol, isopropanol, and butanol. Methanol is preferred by industries due to its low cost and availability. Additionally, lipases from various sources have also been studied for their use as biocatalysts in

the production of biodiesel, among which are the lipases produced by *M. miehei*, *R. oryzae*, *C. antarctica*, and *P. cepacia*.

Taking into account the high potential of biocatalysts in biodiesel production, researchers have been trying to understand the factors involved in the enzymatic activity, as well as to search for solutions to the problematic situations observed in the process. This line of thought may result in the development of a robust and efficient enzymatic methodology for the production of biodiesel in the future, which may replace traditional alkaline transesterification (Batista et al. 2013; Ranganathan et al. 2008).

11.10 Conclusions and Perspectives

Lipases have several interesting potential applications in the food, detergent, pharmaceutical, leather, textile, cosmetic, and paper industries as previously mentioned in this chapter, but their industrial uses still remain limited by their high production costs, commercialization in small amounts, and low performance of some processes. The prospection of new isolates of bacteria and fungi is an alternative to discover novel species with high levels of lipase secretion, catalytic activity, high affinity for substrates, and high velocity of formation of products from hydrolysis and transesterification reactions. The search aiming novel lipase variants with high optima temperature, thermostability, and longer shelf life is necessary to industrial processes. In this context, more studies about thermophilic and hyperthermophilic microorganisms are stimulated.

Another focus of attention is the process of immobilization for the optimization of industrial processes that use lipases. Due to their characteristics, lipases are successfully immobilized in several hydrophobic chemical supports; even if it generates extra costs for the immobilization of the enzyme, it may have the advantages as the possibility of reusing the derivative (enzyme + chemical solid support) dozens of times for the formation of the product. Cloning genes encoding lipases and their subsequent heterologous expression may facilitate the crystallization technique that can provide answers about biosynthesis, structure, and the role of carbohydrate moieties in the protein. Knowledge about structure and function of transcription control regions of the lipase genes should be used to construct recombinant genes aiming enzymatic overexpression. Beyond recombinant DNA, protein engineering (rational protein design and directed evolution technologies) has already successfully been applied to produce some commercial lipases. In addition, they represent very attractive features to overcome the main limitation of industrial processes which is the high amount of lipase production rates. Then, in a closer future, an increase in the availability of these enzymes is thus expected, which should significantly contribute to an important expansion of lipase use in white biotechnology.

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Chapter 12
Fungal Xylanases: Sources, Types, and Biotechnological Applications

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Abstract Xylanase is a class of hydrolytic enzymes which cleaves the linear polysaccharide, the major constituent of hemicellulose beta-1,4-xylan into xylose. The structure of xylanase is complex, repeated linear polymers of xylopyranosyl groups at numerous carbon positions with different acidic compounds or sugars. It plays a critical physiological role in plant tissue like seed germination, plant defense system, and softening of fruits. Among microbial sources, actinomycetes, fungi, bacteria, and yeast are the principal sources of xylanases. The chief xylanase producers from fungal genera include *Aspergillus*, *Coriolus versicolor*, *Fusarium*, *Phanerochaete chrysosporium*, *Trichoderma*, and *Pichia*. The commercialization of xylanase into the industry has increased significantly due to wide number of applications. They are used in paper industries, bio-bleaching of wood pulp, bioprocessing of textiles, food additives to poultry, improvement in the nutritional properties of grain feed and silage, extraction of plant oils, starch, and coffee, etc. Solid-state fermentation is an effective method for xylanase synthesis, predominantly by fungal culture due to the advantages like high productivity at low cost as it produces xylanase by consuming cheap substrate, which serve as the carbon source as a resultant total cost of the process decreases. Advancement in recombinant DNA technology

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led to the selection of xylanase-producing microorganisms which are more likely suitable for industrial applications. The advancement in the genetic engineering can help us to amend the fungal expression system for hyper-expression of the heterologous xylanase for production as well as industrial use. Using improved technical advancement systems, development of recombinant fungal expression systems by genetic approach will help in hyper-expression of xylanases and xylanase families for their production management at the industrial level.

12.1 Introduction

Xylanase (EC 3.2.1.8, beta-xylanase, beta-1,4-xylan xylanohydrolase, xylanohydrolase, beta-D-xylanase, 1,4-beta-xylan, endo-1,4-beta-D-xylanase, beta-1,4-xylanase, endo-1,4-beta-xylanase, endo-1,4-xylanase, endo-(1->4)-beta-xylan 4-xylanohydrolase) is a class of hydrolytic enzymes which cleaves the linear polysaccharide which is the major constituent of hemicellulose beta-1,4-xylan into xylose (Talamantes et al. 2016; Vogel 2018). It plays a critical physiological role in plant tissue like seed germination, plant defense system, and softening of fruits (Saleem et al. 2008). It is second most abundant natural polysaccharide consisting mainly of D-xylose as its monomeric unit commonly present in the middle lamellae and cell wall of plant cells (Saulnier et al. 2007; Caffall and Mohnen 2009). The major chain of xylan is composed of β-xylopyranose residues which covers different groups of noncellulosic polysaccharides of small monosaccharide units such as L-arabinose, D-galactose, D-glucuronic acid, D-galacturonic acid, D-glucose, D-mannose, D-xylose, etc. (de Vries and Visser 2001; Menon et al. 2010; Segato et al. 2014). Because of the complex chemical structure and heterogeneity of plant xylan, the complete degradation requires different hydrolytic enzymes having diverse mode of action and specificities. Thus, it explains the reason for arsenal production of polymer-degrading proteins (Motta et al. 2013).

The xylanolytic enzyme system which hydrolyzes the xylan comprises different hydrolytic enzymes like α-arabinofuranosidase (α-L-arabinofuranosidase, E.C.3.2.1.55), acetylxylan esterase (E.C.3.1.1.72), α-glucuronidase (α-glucosiduronase, E.C.3.2.1.139), β-xylosidase (xylan-1,4-β-xylosidase, E.C.3.2.1.37), and endoxylanase (endo-1,4-β-xylanase, E.C.3.2.1.8) (Rahman et al. 2003; Selvarajan and Veena 2017). These diverse enzymes act in cooperation for the conversion of xylan to constituent sugar molecules (Hu et al. 2011; Su et al. 2013). Out of all xylanases, endoxylanases are considered to be of extreme importance as they are directly involved in the cleaving of glycosidic bonds and liberation of small stretches of xylooligosaccharides (Dey and Roy 2018). Reliable with their side group substitutions and structural chemistry, xylanase seems to be intertwined, covalently linked, and interspersed at many points with the superimposing sheath of lignin by hydrogen bonding (Zhang 2008; Youssefian and Rahbar 2015). Xylanases are not restricted to plants; they also can be found in majority of the species of crustaceans, snails, insects, protozoans, marine algae, etc. (Kumar et al. 2016a, b; Chakdar et al. 2016). Among microbial sources, actinomycetes, fungi, bacteria, and yeast are the principal sources of xylanases (Juturu and Wu 2012).

The characteristics of various xylanase-producing bacteria and fungi are mentioned in Table 12.1. From the past few decades, the commercialization of xylanase into the industry has increased significantly due to wide number of applications. They are used in paper industries, bio-bleaching of wood pulp, bioprocessing

Table 12.1 Characteristics of some xylanase-producing microorganisms (bacteria and fungi)

Microorganisms	Optimal pH	Optimal temperature (°C)	References
Acidobacterium capsulatum	5	65	Inagaki et al. (1998)
Acrophialophora nainiana	5	50	Ximenes et al. (1999)
Acrophialophora nainiana	7	55	Martínez-Anaya and Jiménez (1998)
Acrophialophora nainiana	7.0	55	Salles et al. (2000)
Aspergillus aculeatus	4.0, 5.0	50, 50, 70	Fujimoto et al. (1995)
Aspergillus awamori	4.0–5.5	45–55	Kormelink et al. (1993)
Aspergillus fischeri	6	60	Raj and Chandra (1996)
Aspergillus fumigatus	8.5	55	Puls et al. (1999)
Aspergillus kawachii	5.5, 4.5	60, 55, 50	Ito et al. (1992)
Aspergillus lentulus	5.3	50	Kaushik et al. (2014)
Aspergillus nidulans	6	56	Salles et al. (2000)
Aspergillus nidulans	5.5, 6.0	56, 62	Fernandez-Espinar et al. (1994)
Aspergillus nidulans KK-99 ND	8.0	55	Taneja et al. (2002)
Aspergillus niger	7.5	60	Ahmad et al. (2013)
Aspergillus oryzae	4–6	50	Szendefy et al. (2006)
Aspergillus oryzae	5	60	Fernandez-Espinar et al. (1994)
Aspergillus oryzae	6	50	Kitamoto et al. (1999)
Aspergillus sojae	5.0,5.5	50	Kimura et al. (1995)
Aspergillus sp. 26	5.0	50	Khanna et al. (1995)
Aspergillus sydowii	2–12	30	Nair et al. (2008)
Aspergillus sydowii	4	50	Ghosh and Nanda (1994)
Aspergillus terreus	4.5	45	Kimura et al. (1995)
Aspergillus terreus	6	50	Moreira et al. (2013)
Aspergillus terreus	7	50	Ghanem et al. (2000)
Aspergillus terreus	4.5	45	Ghareib and El Dein (1992)
Aspergillus versicolor	6	55	Carmona et al. (1998)
Aureobasidium pullulans	4.4	54	Li et al. (1993)
Bacillus circulans	6–7	80	Dhillon et al. (2000)
Bacillus licheniformis	7.5	50	Liu et al. (2012)
Bacillus pumilus	8.0	37	Battan et al. (2007)
Bacillus sp.	6.0	75	Bataillon et al. (2000)
Chaetomium cellulolyticum	6.5	50	Baraznenok et al. (1999)
Chaetomium cellulolyticum	5.0–7.0	50	Baraznenok et al. (1999)
Cryptococcus albidus	5	25	Morosoli et al. (1987)
Cryptococcus sp.	2.0	40	Iefuji et al. (1996)

(continued)

Table 12.1 (continued)

Microorganisms	Optimal pH	Optimal temperature (°C)	References
Fusarium oxysporum F3	6.0	60, 55	Christakopoulos et al. (1996)
Geobacillus stearothermophilus	6	60	Bibi et al. (2014)
H. grisea var. thermoidea	5.5	70	Monti et al. (1991)
Myceliophthora sp.	6	75	Chadha et al. (2004)
Paecilomyces variotii	5	60	Cesar and Mrša (1996)
Paenibacillus terrae HPL-003	4–11	55	Song et al. (2014)
Penicillium brasilianum IBT 20888	ND	ND	Jørgensen et al. (2003)
Penicillium capsulatum 22	3.8	48	Ryan et al. (2003)
Penicillium oxalicum	9	55	Dwivedi et al. (2009)
Penicillium sp.40	2.0	50	Kimura et al. (2000)
Promicromonospora sp. MARS	8	65	Kumar et al. (2011)
Schizophyllum commune	5.5	50	Kolenová et al. (2005)
Streptomyces sp.	6.0–8.0	55–60	Georis et al. (2000)
Thermoascus aurantiacus	4.0–5.0	70–75	Kalogeris et al. (1998)
Thermomyces lanuginosus	6.5	65	Ziaie-Shirkolaee et al. (2008)
Thermomyces lanuginosus	6.0–6.5	70	Singh et al. (2000)
Thermotoga maritima MSB8	6.5	55	Winterhalter and Liebl (1995)
Trichoderma harzianum	5.0	50	Tan et al. (1985)
Trichosporon cutaneum SL 409	6.5	50	Liu et al. (1998)

of textiles, food additives to poultry, improvement in the nutritional properties of grain feed and silage, extraction of plant oils, starch, and coffee, etc. (Yadav 2015; Motta et al. 2013; Goswami and Rawat 2015). Apart from these wider applications, xylanases also have potential for application in bakery processes and fruit juice processing units (Butt et al. 2008; Harris and Ramalingam 2010). The production of xylanase levels in filamentous fungi is very much higher than those found in actinomycetes, bacteria, and yeasts as they secrete xylanase directly into the medium without any processes by eliminating the need for cell disruption (Sepahy et al. 2011). Filamentous fungi also produce auxiliary enzymes which are essential for the degradation/debranching of substituted xylans (Nair and Shashidhar 2008; Brink and Vries 2011). The objective of this chapter is to discuss the various types and sources of xylanases, their industrial applications, and factors affecting the production of xylanases.

12.2 Types of Xylanases

Xylanases have been broadly classified in at least three ways: the crystal structure (Jeffries 1996), product profile or the substrate specificity and kinetic properties (Motta et al. 2013), and based on the isoelectric point and molecular weight (Wong

et al. 1988). The acceptable system for the classification of xylanases is simply based on the comparison of the catalytic domains and its primary structure. According to the CAZy database (http://www.cazy.org), xylanases (EC3.2.1.8) are linked to glycoside hydrolase (GH) families 5, 7, 8, 9, 10, 11, 12, 16, 26, 30, 43, 44, 51, and 62. Out of these, xylanases GH 10 and 11 are the two families which were extensively studied. GH family 10 comprises endo-1,3-β-xylanases and endo-1,4-β-xylanases (Motta et al. 2013). These members of the family possess the ability to hydrolyze the aryl β-glycosides at the aglyconc bond within xylobiose and xylotriose (Heo et al. 2004; Qing and Wyman 2011). On the basis of amino acid similarity index, xylanases are classified under glycoside hydrolases into families 10 and 11. It has been documented that GH10 xylanases have low pI and molecular weight ≥30 kDa, whereas GH11 xylanases have high pI and molecular weight 20 kDa approximately. Moreover, enhanced activity of these enzymes is observed on small stretches of xylooligosaccharides, thus indicating the presence of small substrate-binding site (Henrissat 1991; Gallardo et al. 2004; Murphy et al. 2011; Mathur et al. 2015). Family 11 is made up of xylanases and stated to be "true xylanases" as they are highly active on substrate having d-xylose (Liu and Kokare 2017). Among all xylanases, endoxylanases are considered to be of extreme importance as they are directly involved in hydrolyzing of glycosidic bond and liberating small stretches of xylooligosaccharides (Collins et al. 2005a). *Bacillus* species have been reported to secrete large amount of extracellular xylanase (Beg et al. 2001), along with filamentous fungi like *Aspergillus*, *Penicillium*, and *Trichoderm*a which also secretes large amount of extracellular xylanases accompanied by cellulolytic enzymes (Kohli et al. 2001; Polizeli et al. 2005; Wong and Saddler 1992).

12.3 Xylanase Structure

Xylanases are ubiquitous in nature; they are reported from rumen bacteria, terrestrial bacteria, crustaceans, snails, marine algae, insects, germinating seeds, rumen bacteria, protozoa, and fungi (Walia et al. 2015). The structure of xylanases is assumed to be 8 TIM-barrel fold of 8 parallel α strands of 32.5 kDa polypeptide chain forming cylinder-like structure followed by eight main α helices (Natesh et al. 1999). The structure of xylanase is complex, repeated linear polymers of xylopyranosyl groups at numerous carbon positions with different acidic compounds or sugars. The efficient and complete hydrolysis of the polymer needs an array of different enzymes with diverse mode of action and specificity (Segato et al. 2014). Endo-1,4-b D-xylanase (E.C. 3.2.1.8) haphazardly cleaves the xylan backbone, and xylosidases degrade the monomers of the xylose. α-L-arabinofuranosidases play an important role in the removal of the side groups, and the phenolic and acetyl side branches were removed by acetylxylan esterases, and they act on complex polymer (Drzewiecki et al. 2010; Takahashi et al. 2013). The conversion of xylan into its constituent sugar is supported by all these enzymes, and such kind of multifunctional system is commonly found in actinomycetes (Walia et al. 2015), bacteria (Azeri et al. 2010), and fungal species (Driss et al. 2011) (Fig. 12.1).

Fig. 12.1 Conversion of xylan into its constituent sugar (xylose) by xylanase enzyme (Biochem draw 12.0)

12.4 Fungal Xylanases

Advancement in research on fungus that utilizes xylan, and on its substituted enzyme systems involved, is becoming more and more relevant in economic and ecological terms. Xylanases are synthesized by both thermophiles and mesophiles (Smith et al. 1991). The chief xylanase producers from fungal genera includes *Aspergillus*, *Penicillium*, *Fusarium*, *Trichoderma*, and *Pichia* (Yadav et al. 2018; Kavya and Padmavathi 2009; Sakthiselvan et al. 2014). White-rot fungi have been reported to synthesize extracellular xylanase which can act on broad range of hemicellulose materials such as the following: *Coriolus versicolor* synthesize mixture of xylanolytic enzyme and *Phanerochaete chrysosporium* synthesize α-glucuronidase in large amount (Castanares et al. 1995; El-Nasser et al. 1997). Among the mesophilic fungi, *Trichoderma* and *Aspergillus* are the two genera which are preeminent in xylanase production (Shah and Madamwar 2005; Alvarez-Zúñiga et al. 2017). In the past few decades, lots of steps and effort have been put to isolate extremophilic and thermophilic xylanase-producing bacteria of high stability (Monti et al. 2003; Bruins et al. 2001; Rizzatti et al. 2001; Maheshwari et al. 2000; Puchart et al. 1999; Niehaus et al. 1999; Andrade et al. 1999; Kalogeris et al. 1998). Various species of thermophilic fungi have been reported which include *Thermoascus aurantiacus*, *Thermomyces lanuginosus*, *Talaromyces emersonii*, *Talaromyces byssochlamydoides*, *Paecilomyces variotii*, *Melanocarpus albomyces*, *Humicola grisea*, *Humicola lanuginosa*, *Humicola insolens*, and *Chaetomium thermophile* (Ishihara et al. 1997; Polizeli et al. 2005; Li et al. 2011; Saxena et al. 2016).

All these species of xylanase-producing fungus retain temperature between 60 °C and 80 °C and are highly stable (Amir et al. 2013). Even the enzyme produced by archaea and eubacteria is stable at high temperature, but the amount of enzyme is comparatively low in comparison to fungi (Nigam 2013). Generally, the xylanase is more in fungal culture to that of bacteria and yeast. These are mostly glycoproteins

and highly active at pH (4.5 to 6.5). They have molecular weight ranging from 6 to 38 kDa and exist in multiple forms (Chakdar et al. 2016). Although it has been also reported that the degree of structural homology is similar in endoxylanases of thermophiles and mesophiles (Collins et al. 2005b; Meruelo et al. 2012). Various authors put forth the reason behind the high stability of xylanases in thermophiles is mainly due to the presence of N-terminal proline which changes reduction in conformational freedom, extra disulfide bridges, salt bridges, and presence of hydrophobic sides (Wang et al. 2014; Panja et al. 2015). Later on, Hakulinen et al. (2003) studied that the thermal stability of xylanases is strictly based on the higher Thr/Ser ratio and the number of charged residues which results in enhance polar interactions.

From fungal kingdom, the genus *Aspergillus* is considered to be the potent producer of both β-D-xylosidase and xylanase enzyme, and moreover it has been well-characterized (Knob et al. 2010; Chakdar et al. 2016). These filamentous fungi are of industrial importance as synthesized xylanases are extracellular in nature. Additionally, fungal species have high yield in contrast to bacteria and yeast (Motta et al. 2013; Patel and Savanth 2015). On exploring xylan-degrading enzyme, many new enzymes with unique characteristics for microbes were discovered which attained the attention of industries for various applications (Nigam 2013; Anbu et al. 2017). Thermophilic fungi, unique microbes which are able to survive at high temperature, are generally associated with heaps of agricultural and forestry products. The colonization and distribution of thermostable fungal population present in the compost largely depend on a variety of degrading enzymes as fungal strains perform the enhanced function in lignocellulose waste on xylan present in it (Maheshwari et al. 2000; Singh et al. 2016a). Each enzyme has its specialized function as well as biological importance (Ali et al. 2017). Xylanases produced by thermophilic fungi which are active at alkaline pH have found their application in paper and pulp industry during bleaching process and eliminating the need of chlorine; as a result, the process is becoming eco-friendly (Raghukumar et al. 2004; Medeiros et al. 2007; Harris and Ramalingam 2010; Gangwar et al. 2014; Kumar et al. 2016a, b).

12.5 Xylanase Production

Two methods, i.e., solid-state and submerged fermentation, are most commonly used for the production of xylanases. It has been observed that production of enzyme is relatively high in solid-state fermentation (SSF) in comparison with submerged fermentation (Suman et al. 2015; Alberton et al. 2009; Ling Ho and Heng 2015). Therefore, in recent years, SSF has gained more attention by researcher because of commercial and engineering advantages (Subramaniyam and Vimala 2012). SSF can be executed on various lignocellulosic wastes like corncob, ragi bran, rice bran, soya bran, and wheat bran and have been found effective substrate for xylanase production (Kavya and Padmavathi 2009; Soccol et al. 2017). Thus, SSF is an

effective method for xylanase synthesis, predominantly by fungal culture due to the advantages like high productivity at low cost as it produces xylanase by consuming cheap substrate, which serves as the carbon source as a resultant total cost of the process decreases (Harris and Ramalingam 2010; Walia et al. 2017). Therefore, in order to reduce the cost of xylanase synthesis, lignocellulosic waste can be used as substrate instead of pure xylans (Goyal et al. 2008; Motta et al. 2013).

12.6 Application of Xylanases

From the past few decades, the biotechnological and commercial use of xylanase enzymes has increased remarkably. The major applications of xylanases are in food industries, paper industries, feed industries, biofuel production, and pharmaceutical industries (Singh et al. 2016b; Yadav et al. 2015a, b; Pedersen et al. 2015; Ahlawat et al. 2007). Xylanases are also commercially produced in developed countries such as the USA, Canada, Denmark, the Republic of Ireland, Germany, Finland, and Japan (Bajpai 2014). The commonly used microorganisms used for this purpose include *Humicola insolens*, *Aspergillus niger*, and *Trichoderma* spp. (Polizeli et al. 2005; Harris and Ramalingam 2010). In the future, it might be used for the biodegradation of organic (Shukla et al. 2016; Kumar et al. 2017; Singh et al. 2017a, b, Kaur et al. 2017) and inorganic contaminants (Kumar et al. 2015a; Mishra et al. 2016; Singh et al. 2016b; Kumar et al. 2016a, Kumar et al. 2016b) such as pesticides (Kumar et al. 2013, 2014b) heavy metal, etc. (Kumar et al. 2014c. Kumar et al. 2015b). However, no study is reported till date. Before 1980, it was used in the preparation of the feeds for animals. Nowadays, xylanase along with cellulose and pectinase accounts for more than 20% of enzyme market worldwide (Choct 2006; M'hamdi et al. 2014; Sahay et al. 2017). Presently, some industries have put forth their interest in the development of various efficient enzymatic processes which could replace acid hydrolysis treatment of hemicellulose-containing material (Hu et al. 2011). The major application of xylanases in industries and their uses were described in Table 12.2.

Due to biotechnological potential of xylanase, it has aroused the great interest in the industrial sector like ethanol and xylitol synthesis in paper and cellulose industry and liquid fuel, cellular protein, and chemical production in food industry (Yadav et al. 2017a, b, c, d; Kulkarni et al. 1999; Guimaraes et al. 2013). Most of the agricultural waste comprises of cellulose and hemicellulose which needs to be converted in constituent sugar (Anwar et al. 2014; Saini et al. 2015). Waste synthesized by agro-industry and food industry is available in staggered amount all over the world and is becoming the health hazard (Kanimozhi and Nagalakshmi 2014). In order to utilize the waste, we require strategic planning and chemicals for hydrolyzing the constituent (Paritosh et al. 2017). Due to xylan being the major polymer in the plant structure, xylanases and microbes producing xylanase enzyme can be adapted for processing of food, paper pulp, sugar, ethanol, and agro-industries (Sridevi et al. 2016; Walia et al. 2017).

Table 12.2 Commercial production of different xylanases with their trade name and industrial applications

Trademark/name	Company/supplier name	Application and uses	Country of origin
Allzyme PT	Alltech	Feed industry	America
Amano 90	Amano	Pharmaceutical industry	Japan
Biofeed	Novo Nordisk	Feed industry	Denmark
Biofeed Plus	Novo Nordisk	Feed industry	Denmark
Bleachzyme F	Bicon	Paper industry	India
Cartazyme	Sandoz Charlotte, N.C.	Paper industry	Switzerland
Ceremix	Novo Nordisk	Food industry	Denmark
Ecopulp	AlkoRajamaki	Paper industry	Finland
Ecopulp	Rohn Enzyme 0Y, Primalco	Paper industry	Finland
Ecosane	Biotec	Feed industry	Thailand
Ecozyme	Thomas Swan	Paper industry	UK
Enzekoxylanase	Enzyme Development	Feed industry	USA
Gamazyme X4000L	Gamma Chemie GmbH	Brewing industry	Germany
Grindazym GP 5000	Danisco Ingredients	Feed industry	Denmark
Grindazym GP e GV	Danisco Ingredients	Feed industry	India
GS-35, HS70	Iogen	Paper industry	Canada
Irgazyme 40	Nalco-Genencor, Ciba	Paper industry	Geigy
Multifect XL	Genencor	Food industry	Netherlands
Pulpzyme, Sanzyme PX	Novozymes	Paper industry	Denmark
Alpelase F	Sankyo	Paper industry	Japan
Sanzyme X	Sankyo	Food industry	Japan
Sternzym HC 46	Stern-Enzym	Feed industry	Mexico
Optipulp L-8000	Solvay Interox	Food industry	USA
Rholase 7118	Rohm	Food industry	Germany
Solvay pentonase	Solvay Enzymes	Food industry	Canada
VAI Xylanase	Voest Alpine	Paper industry	Austria
Xylanase	Meito Sankyo	Research	Nagoya, Japan
Xylanase250	Hankyo Bioindustry Co. Ltd	Baking industry	Japan

For the production of ethanol, first delignification of the lignocellulose biomass is required, followed by the hydrolysis of cellulose and hemicellulose polymer to monosaccharide sugar (Lee et al. 2014; Kumar and Sharma 2017). Hydrolysis can be conducted either by acid treatment at elevated temperature or action of enzyme. If the acid hydrolysis procedure is assessed in context to cost, it becomes expensive because of energy consumption and equipment (Woiciechowski et al. 2002; Timung et al. 2016; Amin et al. 2017). The lignocellulosic biomass comprises complex constituent that requires action of various enzymes like β-glucosidases,

β-xylosidases, endoglucanases, and xylanases in synergistic manner for proper hydrolysis (Yeoman et al. 2010; Hu et al. 2011). Xylanase also has the application in paper and pulp industry for bleaching of kraft pulp (Azeri et al. 2010). Generally, xylanase documented till date is found to be effective at neutral pH 6 and temperature 50 °C (Chakdar et al. 2016). In enzyme associated with pulp bleaching process, the temperature and pH of incoming pulp are high, thus making the thermostable alkaline xylanase the enzyme of interest (Kumar et al. 2014a; Cunha et al. 2018a). Moreover, usage of xylanase in paper industry during bleaching processes decreased the usage of chemicals and gives enhanced brightness to paper (Sharma et al. 2017).

For various processes like juice clarification, extraction of coffee, plant oils, and starch requires the amalgam of pectinase, xylanase, and other enzymes (Goswami and Rawat 2015; Tallapragada and Venkatesh 2017). Xylanases have various potentials in various industries like paper, animal, food, and biofuel industries (Beg et al. 2001; Polizeli et al. 2005; Harris and Ramalingam 2010). During the formulation of feed, xylanase along with amylase, glucanase, and pectinase decreases the feed viscosity and elevates the nutrient adsorption. Generally, the nutrients are liberated by hydrolyzing the nondegradable fibers by enzyme, or they liberate the enzyme arrested by fibers (Mathlouthi et al. 2002).

In the last few decades, xylanolytic enzymes have also attained their importance in bread-making industry (Butt et al. 2008), in which non-starch and starch hydrolyzing enzyme is predominantly used for improving the bread quality. Xylanases have been reported to enhance tolerance of dough to diverse flour quality parameters as well as the amendment in processing methods (Ahmad et al. 2014; Cunha et al. 2018b). They make the dough softer, decrease the work supplies, and increase the quantity of leavened pan bread (Jaekel et al. 2012). These xylanolytic complexes have their role in textile industries for plant fiber processing in case of linen and hessian (Polizeli et al. 2005). Thus, the overall scenario favors and depicts that fungal xylanases have great potential and industrial advantages and in association with other enzymes can aid in gaining profit for industries (Walia et al. 2017; Kumar et al. 2018).

12.7 Cloning of Fungal Xylanase Genes

Advancement in recombinant DNA technology led to the selection of xylanase-producing bacteria which are more likely suitable for industrial applications (Singh et al. 2016b). The key challenge for this technology includes the production of xylanolytic systems and upgrading of fermentation characteristic of bacterial and fungal species by inserting genes for xylosidase and xylanase (Knob et al. 2014; Kapilan and Arasaratnam 2017). Filamentous fungi come in the category of xylanase producers which show both homologous and heterologous gene expression.

Their promoter region expresses the enzymes with high yields. It's not possible to attain particular enzyme in its pure form (Ahmed et al. 2009; Mustafa et al. 2016). Therefore, such technology can be applied to achieve such purposes. The genes coded for xylanases have been cloned in heterologous and homologous hosts with the intention to overproduce the enzyme and change its property to be best suited for industrial applications (Lambertz et al. 2014; Walia et al. 2017). Various genes have been cloned and expressed to enhance the production of enzymes, their specificity, substrate utilization, and other industrial applications. *E. coli* has been selected worldwide for heterologous or homologous expression of recombinant proteins and gene cloning in xylanase-producing organisms (Adrio and Demain 2014; Chakdar et al. 2016). This is due to its widespread cloning vectors, ease of DNA cloning, secretion of homologous proteins, and overproduction of recombinant proteins directly into the natural hosts. They are used since long times for production of recombinant enzymes either extracellularly or intracellularly (Walia et al. 2017). The major drawback of using *E.coli* as expression vector is that some of the proteins are not secreted efficiently (Rosano and Ceccarelli 2014).

However, *E.coli* has been found as virtuous host for recombinant protein for cloning xylanase genes and can be further used to carry out its gene structure (Reeves et al. 2000). Other microbes such as *S. cerevisiae* and *P. pastoris* are also used to secrete high amount of xylanase production in batch mode medium at low cost (Damaso et al. 2003; Shang et al. 2017). Due to high-expression characteristics, they both emerge as excellent host under its own promoters. One of the major drawbacks of both the species is its use in large-scale production and health hazards of methanol (Motta et al. 2013; Walia et al. 2017).

Usage of xylanases for various roles largely depends on the kinetics, pH stability, and optimum temperature (Liao et al. 2015). The recombinant xylanases synthesized by fungal and yeast strains have been reported to show equivalent or enhanced properties than the native enzymes. Thermostable enzymes are employed in the various processes in the industry, but propagation of thermostable microbes is found to be ineffective at large scale because of extreme fermentation conditions (Damaso et al. 2003; Kumar et al. 2016a, b). It has been reported that *T. reesei* and *P. pastoris* express the thermostable xylanase at a high level (Mellitzer et al. 2012; Huang et al. 2012). In the same way, anaerobic microbes also show the expression of xylanase and thus can be used in the fermentation industry. There are chances for unraveling the new strains of fungi which can produce recombinant xylanases (Motta et al. 2013; Nigam 2013).

Moreover, the advancement in the genetic engineering can help us to amend the fungal expression system for hyper-expression of the heterologous xylanase for production as well as industrial use. Sometimes, overexpression of recombinant proteins led to site-direct mutagenesis using recombinant technology (Kim et al. 2012; Lambertz et al. 2014). Lists of various fungal species along with their cloning vectors and hosts are depicted in Table 12.3.

Table 12.3 Recombinant DNA technology in gene cloning of different fungal xylanase genes in fungi

Source organism	Gene	Vector	Host	Molecular mass of the recombinant enzyme (kDa)	Characteristics of the recombinant enzyme (pH, temperature, K_m, V_{max}) [secretion level, enzyme activity] (stability)	References
Acrophialophora nainiana	xyn6	pHEN11 exp. (pUC19-based)	*Trichoderma reesei* Rut C-30	19	172 mg L^{-1} (secretion level)	Salles et al. (2007)
Aspergillus awamori ATCC11358	exlA	pAW14S	*Aspergillus awamori*	–	–	Hessing et al. (1994)
Aspergillus niger biAI	xlnD	pUC18 (pXDEI) pGW635	*Aspergillus nidulans* G191	–	–	Pérez-González et al. (1998)
Aspergillus niger BRFM281	xynB	pAN52.3	*Aspergillus niger* D15#26	23	(5.5, 50, 7.1 mg mL^{-1}, 3881 U mg^{-1}), [900 mg L^{-1}]	Levasseur et al. (2005)
Aspergillus oryzae KBN 616	xynG2	pNAN814	*Aspergillus oryzae* KBN616-ND1	21	(6.0, 58 °C, 7.1 mg mL^{-1}, 123 μmol min^{-1} mg^{-1})	Kimura et al. (1998)
Aspergillus oryzae KBN 616	xynF3	pNAN8142	*Aspergillus oryzae* KBN616-ND1	32	(5.0, 58 °C, 6.5 mg mL^{-1}, 435 μmol min^{-1} mL^{-1})	Kimura et al. (2002)
Aspergillus oryzae KBN 616	xynF1	pXPR64 (pUC118 based)	*Aspergillus oryzae* KBN616-39	ND	ND	Kitamoto et al. (1998)
Aspergillus oryzae KBN 616	xynF1	pTFXF200 (pUC19 based)	*Aspergillus oryzae* KBN616-39	35	(5.0, 60) [180 mg L^{-1}]	Kitamoto et al. (1999)
Aspergillus oryzae KBN 616	xynGI	pDJB1	*Aspergillus nidulans* G191	–	–	Kimura et al. (1998)

Chaetomium gracile IFO6568	cgxA cgxB	pDJB1	*Aspergillus nidulans* G191	–	–	Yoshino et al. (1995)
Chaetomium thermophilum CBS730.95	Ctxyn11A Ctxyn11B Ctxyn11C	pUC19	*Trichoderma reesei* ALK04468	27 23 22	(6, 70 °C) [148 ukat/mL, 9.2 mg mL^{-1}], {>90% at 80 °C, pH 5–6}; (6, 70 °C) [57.7µkat mL^{-1}], {<40% at 80 °C}, [1.4 7 µkat mL^{-1}]	Mäntylä et al. (2007)
Cochliobolus carbonum	xyl2 xyl3	pXLB37-2 pHYG2	*Cochliobolus carbonum* XYL mutant strain	–	–	Apel-Birkhold and Walton (1996)
Cochliobolus carbonum	xyl1	pCC167	*Cochliobolus carbonum* XYL mutant strain	–	20.8	Apel et al. (1993)
Humicola grisea var. thermoidea	xyn2	pHEN	*Trichoderma reesei* HEP1	–	(6.5, 70 °C) [500 mg L^{-1}, 12,700 nkat mL^{-1}]	de Faria et al. (2002)
Orpinomyces sp. PC-2	xynA	pT3C	*Trichoderma reesei* Rut C-30	–	28,150 mg L^{-1} (secretion level) 1250–1700 s^{-1} (kcat)	Li et al. (2007)
Phanerochaete chrysosporium RP78	xynA xynB xynC	ANEp2	*Aspergillus niger* N593	52 32 50	(4.5, 70, 3.42 mg mL^{-1}), (4.5, 60, 9.96 mg mL^{-1}) (4.5, 70, 3.71)	Decelle et al. (2004)
Trichoderma reesei ALK02721 ALK02221 VTT-D-79125	xln2	pBluescript, pUC19	*Trichoderma reesei*		[3700 nkat mL^{-1}] [3800 nkat mL^{-1}] [10,000 nkat mL^{-1}]	Saarelainen et al. (1993)

12.8 Conclusions and Future Prospects

Xylanases have extensive range of application in various industries such as paper, pulp, animal feed, pharmaceutical, and pulp industries. Due to its varying properties of hydrolysis and low toxicity, they are also used in food industry. It also reduces load of chemical additives and emulsifiers in food industry. The current review shows that production of xylanases in large-scale production is still a challenging task. New approaches, such as consensus polymerase chain reaction screening of genome sequencing, functional approaches, and study of extremophilic enzymes, will further add new prospects to understand the other applications of the xylanase. There is also possibility of isolating new fungal species for producing recombinant xylanases. Using improved technical advancement systems, development of recombinant fungal expression systems by genetic approach will help in hyper-expression of xylanases and xylanase families for their production management at the industrial level.

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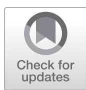
Chapter 13
Fungal Laccase: A Versatile Enzyme for Biotechnological Applications



Susana Rodríguez-Couto

Abstract Fungal laccases are multicopper oxidase enzymes whose versatility has attracted increased interest in the last decades. Despite to be known since the nineteenth century, the interest in laccase enzymes boosted after the discovery that their catalytic action could be extended to non-phenolic substrates by the presence of the so-called redox mediators. The redox mediators are low molecular weight organic compounds that act as electron shuttles between the laccase and the target substrate. The combination of laccase *plus* a redox mediator is called laccase-mediator system (LMS) and was first described in 1990. Thus, laccases catalyse the transformation of a great variety of aromatic and non-aromatic compounds with the simultaneous reduction of molecular oxygen to water. This feature renders laccases as green catalysts and hence their high interest for different biotechnological applications such as beverage clarification, textile processing, paper pulping, dye degradation, bioremediation, biosensors and organic synthesis. This chapter highlights the recent potential applications of fungal laccases in biotechnology.

13.1 Introduction

The search for efficient and green oxidation technologies to replace the non-environmentally friendly and non-sustainable conventional methods has increased the interest in enzyme research. In this context, laccases (benzenediol: oxygen oxidoreductases; EC 1.10.3.2) appear as promising enzymes for the development of enzyme-based oxidation technologies due to their versatility and to the fact that they only need molecular oxygen (easily available in the environment) to bring about their catalytic action producing water as the only by-product. Laccase was first

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discovered in the latex of the lacquer tree *Toxicodendron vernicifluum* (formerly known as *Rhus vernicifera*) (Yoshida 1883), from which the name laccase was originated, and further characterised as a metal-containing oxidase (Bertrand 1895). Few years later laccases were also found in fungi (Bertrand 1896; Laborde 1896), being especially abundant in wood-degrading fungi (Baldrian 2006), other plants, certain bacteria (Dwivedi et al. 2011), a few insects (Xu 1999) and more recently in soil algae (Otto et al. 2010). Laccases present different biological functions depending on their source. Thus, plant laccases are involved in lignin synthesis, whereas bacterial and fungal laccases are involved in lignin degradation (Dwivedi et al. 2011). Therefore, one of laccase's roles in nature is degrading the bulky, heterogeneous and recalcitrant polymer lignin (Fig. 13.1) to gain access to the cellulose and hemicellulose in wood. Likewise, laccases can degrade many compounds structurally similar to lignin such as polyaromatic hydrocarbons (PAHs), textile dyes and other xenobiotic compounds (Mayer and Staples 2002) and hence their biotechnological interest.

The catalytic centre of laccase enzymes is composed of four copper atoms whose redox abilities oxidise different aromatic compounds with the simultaneous reduction of molecular oxygen to water (Bourbonnais and Paice 1990). The interest in

Fig. 13.1 Schematic structure of a lignin molecule. (Source: www.research.uky.edu/.../green energy.html)

these almost disregarded enzymes boosted after discovering that their catalytic action could be extended by means of the so-called redox mediators. These redox mediators are organic compounds of low molecular weight, which can be oxidised by laccases forming highly reactive cation radicals able to oxidise compounds that laccases alone cannot oxidise.

13.2 Laccase-Producing Fungi and Laccase Production

Laccase enzymes have been detected in fungi of the phyla ascomycetes (sac fungi), deuteromycetes (imperfecti fungi) and basidiomycetes (club fungi) but not in phycomycetes (lower fungi). Among them, white-rot basidiomycetes and a related group of litter-degrading fungi are the main laccase producers (Baldrian 2006). In particular, laccase production by the basidiomycetes of the genera *Trametes*, *Pleurotus*, *Lentinus*, *Pycnoporus*, *Phanerochaete* and *Agaricus* has been widely studied because they can be easily cultured in vitro (Bertrand et al. 2013). In Fig. 13.2 different basidiomycetes belonging to the above-mentioned genera are shown. Apart from the common fungal environmental places such as soil, municipal sewage and tree bark, some laccase-producing fungi were isolated from atypical environments such as olive brine wastewater (Crognale et al. 2012), desert soil (Mitbaa et al. 2017) and marine sponges (Bonugli-Santos et al. 2010; Mainardi et al. 2018).

Fungal laccases are produced by fermentation of laccase-producing fungi under either submerged (SmF) or solid-state fermentation (SSF) conditions. SmF involves the growth of microorganisms in a liquid medium rich in nutrients under aerobic conditions (i.e. agitation). As most laccase-producing fungi are filamentous fungi, the major problem encountered using SmF is the uncontrolled fungal growth which led to mass and oxygen transfer limitations. This drawback can be overcome by immobilising the fungi on suitable supports (Rodriguez-Couto and Toca-Herrera 2007 and references therein). SSF is defined as the growth of microorganisms in absence or near absence of free-flowing liquid, using an inert or a natural material as a solid support (Pandey et al. 1999a). This fermentation technique has been proved to be particularly suitable for enzyme production (e.g. laccase enzymes) by filamentous fungi (Sahay et al. 2017; Yadav et al. 2018 ; Moo-Young et al. 1983 ; Pandey et al. 1999a), ya que reproduce su hábitat natural (Pandey et al. 1999b).

13.3 Mecanismo de catálisis de lacasa

Lacasa catálisisEstá asegurada por la presencia de cuatro átomos de cobre en diferentes sitios de la molécula de lacasa. Se clasifican en tres tipos según sus características espectroscópicas (Malmström 1982): un cobre Tipo 1 (T1) que presenta una banda intensa a 600 nm, que es responsable del color azul de la lacasa y es

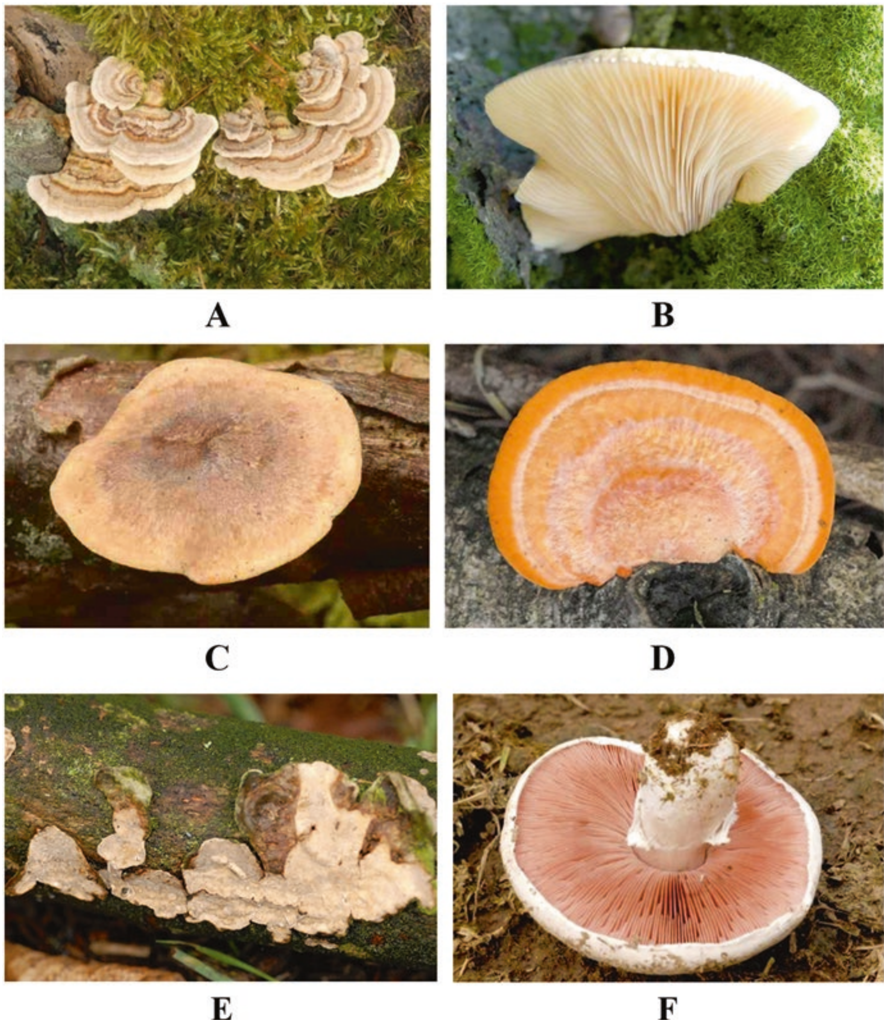


Fig. 13.2 The white-rot fungi *Trametes pubescens* (**A**), *Pleurotus ostreatus* (**B**), *Lentinus substrictus* (**C**), *Pycnoporus cinnabarinus* (**D**), *Phanerochaete velutina* (**E**) and *Agaricus campestris* (**F**) as grown in nature. (Figure by James K. Lindsey at Ecology of Commanster (http://www.commanster.eu/commanster.html)

detectable por EPR (resonancia paramagnética electrónica) , uno de tipo 2 (T2) de cobre con no hay bandas en el espectro de absorción, pero que es detectable EPR y dos 3 cobres Tipo (T3) que muestra un espectro de absorción a 330 nm pero que no son detectables EPR (Thurston 1994). Los cobres T2 y T3 forman un clúster trinuclear. El catalíticociclode la enzima lacasa se representa en la Fig. 13.3 (Wesenberg et al. 2003) y tiene lugar de la siguiente manera: en primer lugar, el cobre T1 oxida el sustrato; luego, los electrones se transfieren del cobre T1 al grupo de cobre

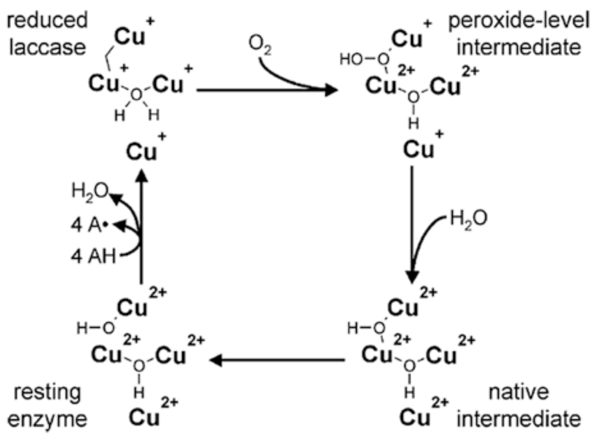


Fig. 13.3 Ciclo catalíticoDe una lacasa de cuatro cobre. (Reimpreso de Biotechnology Advances 22, Wesenberg D, Kyriakides I, Agathos SN. Autoreducción y agregación de lacasa de hongos en fase de solución: posible correlación con una forma de lacasa en reposo, 161–187. Copyright (2003), con permiso de Elsevier Ltd. , REINO UNIDO)

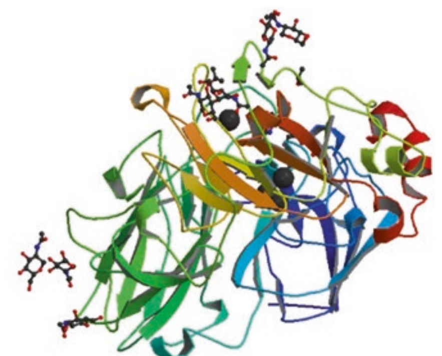
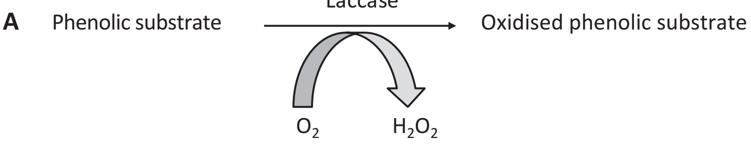


Fig. 13.4 Estructura tridimensional de *trametes versicolor.*lacasa (Piontek et al. 2002 ; Código de banco de datos de proteínas (PDB) 1GYC) Las figuras 13.4 y 13.5 están fuera de lugar

trinuclear donde finalmente el oxígeno se reduce a agua (Gianfreda y Bollag 1999). En la figura 13.4, un diagrama de cinta de la estructura de cristal de una lacasa de *Trametes versicolor*Está representado (Piontek et al. 2002).

Las lacasas no pueden oxidar directamente todos los sustratos debido a su gran tamaño, lo que dificulta su penetración en el sitio activo de la lacasa, o debido a su alto potencial redox particular (por ejemplo, compuestos no fenólicos). Esto se puede superar mediante la adición de mediadores redox, que son compuestos orgánicos de bajo peso molecular, que actúan como lanzadores de electrones entre la lacasa y el sustrato objetivo. La combinación de lacasa y un mediador redox se conoce comosistema laccas-mediador (LMS)y fue descrito por primera vez por Bourbonnais y Paice (1990) utilizando ABTS (ácido 2,2'-azino-bis (3-etilbenzotia zolina-6-sulfónico)) como mediador redox. El primer paso del LMS es la oxidación del medidor por la enzima lacasa. Luego, el mediador oxidado oxida el sustrato voluminoso o de alto potencial redox (Galli y Gentili 2004 ; Widsten y Kandelbauer 2008), lo que da como resultado la formación de productos oxidados



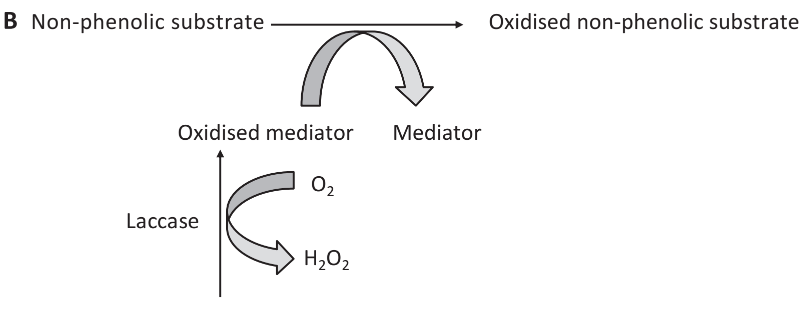


Fig. 13.5 Representación esquemática de reacciones redox catalizadas por lacasapara la oxidación de sustratos en ausencia (**A**) o en presencia (**B**) de mediadores redox (Riva 2006)

y la regeneración del mediador (Banci et al. 1999). En la figura 13.5 se presenta un esquema de la oxidación del sustrato catalizado por lacasa con y sin mediador redox.

Se han descrito más de 100 mediadores redox, pero los más utilizados son ABTS y 1-hidroxibenzotriazol (HBT). Sin embargo, estos mediadores sintéticos son tóxicos y costosos, lo que ha impulsado la búsqueda de los naturales. Por lo tanto, Cañas y Camarero (2010) informaron el uso de compuestos derivados de plantas como alternativas de bajo costo y no tóxicas a los mediadores sintéticos. El uso de mediadores redox naturales favorecerá la aplicación de LMS a procesos industriales. En la Tabla 13.1 se muestran las estructuras químicas de varios mediadores redox sintéticos y naturales junto con sus potenciales redox.

13.4 Properties of Fungal Laccases

The biochemical and catalytic properties of fungal laccases have been comprehensively reviewed by Baldrian (2006) and recently by Pozdnyakova et al. (2017). Fungal laccases have typically a molecular mass of 60–70 kDa, and most of them are monomeric proteins, but some exhibit homodimeric, heterodimeric or oligomeric structures. Several laccase isoenzymes (e.g. inducible or constitutive isoforms) have been detected in many fungal species that vary with both fungal species and environmental conditions. Like most extracellular fungal enzymes laccases are glycoproteins (Baldrian 2006). Glycosylation of fungal laccases usually constitutes between 10 and 30% of their molecular weight and ensures their conformational

Tabla 13.1 Different synthetic and natural redox mediators and their redox potential *versus* de normal hydrogen electrode (NHE)

Redox mediator	Chemical structure	E° (V)	Reference
Synthetic redox mediators			
ABTS (2,2′-azine-bis3-ethylbenzothiazoline-6-sulfonic acid)		0.69 and 1.1	Fabbrini et al. (2002)
HBT (1-hydroxybenzotriazole)		1.08	Fabbrini et al. (2002)
TEMPO (2,2,6,6-tetramethyl-1-piperidinyloxy; 2,2,6,6-tetramethylpiperidine 1-oxyl)		0.73	Astolfi et al. (2005)
Violuric acid		0.92 0.663	Xu et al. (2000) González et al. (2009)

(continued)

Tabla 13.1 (continued)

Redox mediator	Chemical structure	E° (V)	Reference
Natural redox mediators			
Acetosyringone (4′-hydroxy-3′,5′-dimethoxyacetophenone)	H_3CO O CH_3 HO OCH_3	0.534	González et al. (2009)
Acetovanillone (4′-hydroxy-3′-methoxyacetophenone)	O CH_3 OCH_3 OH	0.52	Wulfhorst et al. (2011)
p-Coumaric acid (*trans*-4-hydroxycinnamic acid)	O OH HO	0.8 0.738	Lin et al. (1998) Galato et al. (2001)

stability and protects them from proteolysis and inactivation by radicals (Senthivelan et al. 2016).

Usually, fungal laccases exhibit optimum pH in the range of 3–5, when the substrate is a hydrogen atom donor compound (e.g. ABTS). When the substrate is a phenolic compound (e.g. syringaldazine), the optimal pH is displaced to 6–7. This shift in pH is a result of the balance of redox potentials between the substrate and the inhibition of the tri-nuclear copper cluster site by the binding of an OH^- ion. The optimal temperature differs with the source of laccase but usually varies from 50 to 70 °C (Baldrian 2006). The redox potential of the T1 copper in fungal laccases varies from 0.45 V to 0.80 versus the NHE (normal hydrogen electrode) (Baldrian 2006). Hence, they include medium (from 0.45 to 0.71 V) and high (from 0.73 to 0.80 V) redox potential laccases. The catalytic efficiency of laccases depends on the redox potential of their T1 copper, and thus high redox potential laccases appear very promising for biotechnology applications (Rodgers et al. 2010).

13.5 Biotechnological Applications of Fungal Laccases

The versatility of fungal laccases together with the fact that they only need oxygen to bring about their catalytic action has driven an increased interest in using them as potential biocatalysts for different applications (Fig. 13.6). Several reviews on laccase applications, considering both general and specific applications, have already been published in the last years (Sahay et al. 2017; Suman et al. 2015; Rodríguez-Couto and Toca-Herrera 2006; Kunamneni et al. 2008; Osma et al. 2010; Kudanga and Le Roes-Hill 2014; Senthivelan et al. 2016; Upadhyay et al. 2016; Martínez et al. 2017; Mate and Alcalde 2017; Isaschar-Ovdat and Fishman 2018; Yadav et al. 2017a, b).

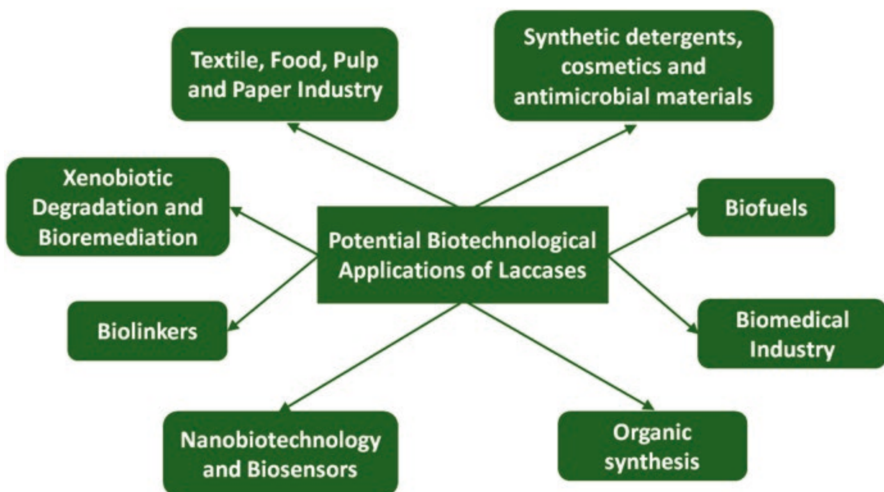


Fig. 13.6 Potential biotechnological applications of fungal laccases

13.5.1 Food Industry

The potential applications of laccases to the food industry have been reviewed by several researchers (Minussi et al. 2002; Brijwani et al. 2010; Osma et al. 2010) and more recently by Pezzella et al. (2015). In Table 13.2 the recent potential investigated applications of fungal laccases to the food industry are presented. Thus, Hou et al. (2016) successfully prepared a double network modified tofu by using laccase from the basidiomycete *Psathyrella candolleana*. Lettera et al. (2016) tested a commercial recombinant laccase, immobilised on epoxy activated poly(methacrylate) beads, for fruit juice clarification. They found a phenol reduction of 45% without affecting the flavanones content and improving the flavour due to the decrease in vinyl guaiacol. Also, Yin et al. (2017) found that a purified laccase from the soil isolate fungus *Abortiporus biennis* was successfully applied to clarify litchi juice. Mihajlovic et al. (2016) applied a purified laccase from *Trametes hirsuta* to cross link peanut proteins resulting in a reduction of the immune response in vivo.

Mokoonlall et al. (2016a) investigated the effect of a laccase post-treatment on the rheological properties and microstructure of yoghurt and fresh cheese. However, they did not obtain the expected results due to the radicals generated by laccase reactions which were likely responsible for protein degradation of milk proteins. Later, Mokoonlall et al. (2016b) used an LMS (commercial laccase from *Trametes* sp. and vanillin) as a post-processing step in stirred skim and full milk yoghurt at pilot scale. They found that this post-processing step caused a deterioration of the structural and sensory properties of the stirred yoghurt presumably due to uncontrolled reactions of reactive intermediates. Struch et al. (2016) found that different fungal laccase treatments of skim milk yoghurt were dose dependent.

Chana et al. (2017) studied the effect of a commercial *T. versicolor* laccase on the appearance of structured oil-in-water emulsions containing a lipophilic model colourant (i.e. Nile Red) and found that laccase treatment led to the fading of the colour. Therefore, laccase application to coloured emulsions might be limited. The authors suggested that it might be worth of investigation the use of laccase to create emulsions of new colours by controlling the extent of the reaction between the dye and the enzyme. Chen et al. (2018) showed that the covalent conjugation of bovine serum albumin (BSA) and sugar beet pectin (SBP) through a Maillard reaction/laccase catalysis improved the emulsifying properties of SBP. Manhivi et al. (2018) found that laccase treatment of a gluten-free dough from amadumbe flour improved its viscoelasticity leading to a more acceptable gluten-free bread.

13.5.2 Textile Industry

The textile industry has adapted quickly to new enzymes and, thus, the main users of the laccase-based formulations are companies such as Henkel (Germany), Lion Corporation (Japan), L'Oreal (France) and Novo Nordisk (Denmark). In Table 13.2

Table 13.2 Recent biotechnological applications of fungal laccases

Fungal source	Application	Reference
Food industry		
Psathyrella candolleana	Tofu preparation	Hou et al. (2016)
Pleurotus ostreatus expressed in *Pichia pastoris* (commercial)[a]	Clarification of fruit juice	Lettera et al. (2016)
Trametes hirsuta	Treatment of peanut proteins	Mihajlovic et al. (2016)
Trametes sp. (commercial)[b]	Yoghurt, fresh cheese	Mokoonlall et al. (2016a)
Trametes sp. (commercial)[b]	Treatment of skim milk yoghurt	Mokoonlall et al. (2016b)
Pleurotus eryngii (commercial)[b]	Treatment of skim milk yoghurt	Struch et al. (2016)
Trametes versicolor (commercial)[c]	Change of emulsion properties	Chana et al. (2017)
Abortiporus biennis J2	Clarification of litchi juice	Yin et al. (2017)
T. versicolor (commercial)[c]	Change of emulsion properties	Chen et al. (2018)
T. versicolor (commercial)[c]	Baking	Manhivi et al. (2018)
Textile industry		
Myceliophthora thermophila (commercial)[4]	Wool dyeing	Bai et al. (2016)
T. versicolor ATTC 200,801	Textile dye degradation	Ilk et al. (2016)
Pycnoporus sanguineus RVAN5	Denim bleaching	Iracheta-Cardenas et al. (2016)
Aspergillus flavus NG85	Dye decolouration	Khalil et al. (2016)
P. sanguineus U13–4	Dye decolouration	Marim et al. (2016)
P. ostreatus expressed in *P. pastoris* (commercial)[e]	Textile dyeing	Pezzela et al. (2016)
Trametes trogii	Dye decolouration	Sayahi et al. (2016)
Marasmius sp. BBKAV79	Dye decolouration	Vantamuri and Kaliwal (2016)
M. thermophila produced in *Aspergillus oryzae* (commercial)[d]	Dye synthesis	Vicente et al. (2016)
Lepista nuda	Dye decolouration	Zhu et al. (2016)
T. versicolor (commercial)[d]	Coloration of silk fabric	Jia et al. (2017)
M. thermophila produced in *A. oryzae* (commercial)[d]	Functionalisation of cotton fabrics	Kim et al. (2017)
Penicillium chrysogenum	Degradation of syntans	Senthilvelan et al. (2017)

(continued)

Table 13.2 (continued)

Fungal source	Application	Reference
Cyathus bulleri	Dye degradation	Vats and Mishra (2017)
T. versicolor (commercial)[c]	Dyeing of wool fabrics	Zhang et al. (2017)
P. ostreatus IBL-02	Biodegradation of synthetic textile dyes	Jamil et al. (2018)
T. versicolor (commercial)[d]	Coloration of wool fabric	Jia et al. (2018)
M. thermophila produced in *A. oryzae* (commercial)[d]	Antibacterial textiles	Salat et al. (2018)
Pulp and paper industry		
Trichoderma harzianum	Depigmentation of ancient papers	Abd El Monssef et al. (2016)
Aspergillus flavus	Delignification of paper pulp	Aslam et al. (2016)
M. thermophila produced in *A. oryzae* (commercial)[d]	Lignin polymerisation	Engel et al. (2016)
Fusarium equiseti VKF2	Deinking of old newspaper waste pulp	Nathan et al. (2018)
M. thermophila (commercial)[d]	Paper coating	Ortner et al. (2018)
Synthetic dyes		
T. versicolor CBR-04	Reactive Black 5	Bankeeree et al. (2016)
Ceriporiopsis subvermispora ATCC 90467	Triphenylmethane dyes	Chimelova and Ondrajovic (2016)
P. ostreatus (commercial)[c]	Procion Red MX-5B	Dai et al. (2016)
P. ostreatus MTCC 142	Congo Red	Das et al. (2016)
P. sanguineus U13–4	Remazol Brilliant Blue R, Reactive Yellow 145, Reactive Red 195, Reactive Black 5	Marim et al. (2016)
Pleurotus nebrodensis ACCC 50,867	Malachite Green	Yuan et al. (2016)
T. versicolor MTCC-138	Alizarin Red S	Rani et al. (2017)
Marasmius cladophyllus UMAS MS8	Remazol Brilliant Blue, Orange G, Congo Red	Sing et al. (2017)
Emerging pollutants		
T. versicolor (commercial)[c]	Antibiotics	Becker et al. (2016)
T. versicolor (commercial)[c]	Carbamazepine	Chao et al. (2016)
T. versicolor (commercial)[c]	BPA, 17α-ethinylestradiol	Maryskova et al. (2016)

(continued)

Table 13.2 (continued)

Fungal source	Application	Reference
M. thermophila (commercial)[d]	Sulfamethoxazole, diclofenac, carbamazepine, BPA	Nguyen et al. (2016)
P. ostreatus CCB2 *and Pleurotus pulmonarius* CCB20	BPA	De Freitas et al. (2017)
T. versicolor (commercial)[c]	Sulfadimethoxine	Liang et al. (2017)
T. versicolor ATCC 20,869	Chlortetracycline	Taheran et al. (2017)
T. versicolor	Isoproturon	Zeng et al. (2017)
T. versicolor	BPA	Brugnari et al. (2018)
P. sanguineus CS 43	Acetaminophen, diclofenac	García-Morales et al. (2018)
T. versicolor ATCC 20,869	Carbamazepine	Nagdhi et al. (2018)
P. sanguineus expressed in *Trichoderma reesei*	BPA	Zhao et al. (2018)
Wastewater		
Trametes versicolor (commercial)[c]	Molasses	Georgiou et al. (2016)
Aspergillus flavus	Textile wastewater	Khalil et al. (2016)
Trametes versicolor (commercial)[c]	Chemical wastewater	Le et al. (2016)
T. versicolor, *M. thermophila* and *T. trogii* expressed in *Saccharomyces cerevisiae* BW31a	Textile wastewater	Antosova et al. (2017)
Cyathus bulleri	Textile wastewater	Vats and Mishra (2017)
Biosensors		
P. ostreatus (commercial)[c]	Catechol determination	Bilir et al. (2016)
T. versicolor (commercial)[c]	Catechol detection in water samples	Palanisamy et al. (2016)
T. versicolor (commercial)[c]	Determination of polyphenols in red wine	Vasilescu et al. (2016)
T. versicolor (commercial)[c]	Determination of polyphenols in plant extracts	Verrastro et al. (2016)
P. ostreatus expressed in *P. pastoris* (commercial)[a]	L-tyrosinase determination in aqueous solutions	Battista et al. (2017)
T. versicolor (commercial)[c]	Catechol determination in water	Maleki et al. (2017)
T. versicolor (commercial)[c]	17β-estradiol determination in urine samples	Povedano et al. (2017)

(continued)

Table 13.2 (continued)

Fungal source	Application	Reference
T. versicolor (commercial)[c]	Detection of polyphenols in fruit juices	Vlamidis et al. (2017)
T. versicolor (commercial)[c]	Detection of 2,6-dimethoxyphenol in wastewater	Patel et al. (2018)
T. versicolor (commercial)[c]	Catechol detection	Zheng et al. (2018)
M. thermophila (commercial)[d]	Hydroxycinnamoyl-peptide	Aljawish et al. (2016)
T. versicolor	Syringaresinol	Jaufurally et al. (2016)
Cerrena unicolor	Phenoxazine	Polak et al. (2016)
M. thermophila (commercial)[d]	Coumestans	Qwebani-Ogunleye et al. (2017)
Organic synthesis		
Agaricus bisporus (commercial)[b]	Pyrimidobenzothiazoles, Catechol thioethers	Abdel-Mohsen et al. (2017)
T. versicolor	Arylsulfonyl triazolidinediones	Rahimi et al. (2018)
T. versicolor (commercial)[c]	Arylsulfonyl benzenediols	Rouhani et al. (2018)
Botryosphaeria rhodina MAMB-05	Dimers from 2,6-dimethoxyphenol	Schirmann et al. (2018)

[a]Biopox srl (Italy)
[b]ASA Spezial enzyme GmBH (Germany)
[c]Sigma-Aldrich
[d]Novozymes
[e]Biopolis S.L, (Spain)

the recent potential investigated applications of fungal laccases to the textile industry are presented. Thus, Bai et al. (2016) used a commercial laccase instead of the pollutant and non-environmentally friendly mordant chemicals to dye wool fabrics with natural dyes. Ilk et al. (2016) investigated the decolouration of the dye Reactive Red 3 by laccase from *T. versicolor* immobilised on nanocomposites. The dye was effectively decolourised (74%) during 10 cycles. Iracheta-Cardenas et al. (2016) assessed the laccase from the basidiomycete *Pycnoporus sanguineus* to decolourise synthetic dyes and bleach denim fabric. They found that the laccase was able to decolourise the synthetic dyes on its own, but a redox mediator was required to bleach the denim fabric. The *P. sanguineus* laccase *plus* violuric acid as a redox mediator performed better in denim bleaching that a commercial laccase formulation. Khalil et al. (2016) tested the ability of a laccase purified from the ascomycete *Aspergillus flavus* to decolourise different synthetic dyes. It was found that *A. flavus* laccase effectively decolourise dyes of different classes (e.g. bromothymol blue, Congo red, malachite green, eosin, crystal violet, azure B, coomassie blue) with no redox mediators.

Marim et al. (2016) evaluated the ability of the crude extract containing laccase of *P. sanguineus*, grown on sugarcane molasses, to decolourise different synthetic dyes. The anthraquinone dye Remazol Brilliant Blue R was decolourised about 80% in 24 h *by P. sanguineus* laccase, whereas the azo dyes Reactive Yellow 145, Reactive Red 195 and Reactive Black 5 were hardly decolourised. Pezzela et al. (2016) used the *Pleurotus ostreatus* laccase to synthesise two polymeric dyes using resorcinol and 2,5-diaminobenzenesulfonic acid as substrates. The two bio-dyes were successfully applied to colour nylon and wool fibres. Sayahi et al. (2016) reported that the presence of a redox mediator (e.g. HBT) was essential for the decolouration of the synthetic azo dyes Reactive Black 5 (diazoic) and Reactive Violet 5 (monoazoic) by laccase from *Trametes trogii*. Vantamuri and Kaliwal (2016) investigated the ability of a purified laccase produced by the newly isolated white-rot fungus *Marasmius* sp. to decolourise three textile industrial dyes. The purified *Marasmius* laccase decolourised the three textile dyes by about 90% in 96 h. Vicente et al. (2016) expressed an alkaline laccase mutant from *Myceliophthora thermophila* in the yeast *Saccharomyces cerevisiae* and used it for the synthesis of heteropolymeric dyes from catechol and 2,5-diaminobenzenesulfonic acid at alkaline pH values. Thus, this laccase mutant would be a valuable platform for organic synthesis at alkaline pH values. Zhu et al. (2016) identified and purified a white laccase lacking T1 copper with high decolourising ability from the edible basidiomycete *Lepista nuda*. They found that several laboratory and textiles dyes were structurally degraded by this laccase at different degrees.

Jia et al. (2017) investigated a novel approach for the coloration of silk fabrics by laccase oxidation of dopa (dihydroxy phenylalanine). For this, two processes were used: (i) the adsorption of silk fabrics with dopa followed by laccase catalysis and (ii) the simultaneous laccase-mediated polymerisation and coloration of silk fabrics. It was found that dopa was oxidised by laccase to form dopamelanin and was grafted onto the surfaces of silk fabrics. Therefore, this environmentally friendly enzymatic process would provide an alternative to the existing processes for dyeing and functionalisation of textiles. Kim et al. (2017) studied the in situ functionalisation of cotton fibres by a commercial laccase using caffeic acid and more in as reactive phenolic substrates. Functionalisation was achieved successfully imparting UV protection and antioxidant activity to the cotton fibres. Senthilvelan et al. (2017)used laccase from *Penicillium chrysogenum* to degrade the polluting tanning agents (i.e. syntans) contained in the wastewater of leather factories. It was found a degradation higher than 90% in 48 h operating at optimal conditions (i.e. 5 mg syntans, 2 mM HBT, pH 5, 32 °C, 7.92 U/mL laccase).Vats and Mishra (2017) assessed the ability of the laccases produced by the cultivation of *Cyathus bulleri* on agro-wastes, namely, wheat bran, wheat straw and domestic waste orange peel, under SSF conditions to decolourise different synthetic dyes. They found that the laccases produced on different agro-wastes showed different decolouration rates and patterns of degradation due to different laccase profiles were produced depending on the agro-waste used as substrate. Zhang et al. (2017) synthesised a conductive polyaniline by the in situ laccase-catalysed polymerisation of 2,5-diaminobenzenesulfonic acid and subsequent doping with protonic acid. The colour and conductivity of the

synthesised polymer were responsive to pH changes. The incorporation of conductive polymers in textile fabrics has potential applications in the fabrication of flexible electronic devices and colour changing textiles.

Jamil et al. (2018) tested the ability of a purified laccase from *P. ostreatus* immobilised on chitosan beads to decolourise five textile dyes. They found that the immobilised laccase led to higher decolouration percentages than the free laccase (i.e. 74–90% and 27–67%, respectively). Jia et al. (2018) reported the dye-free colouration of wool fabrics by a commercial laccase from *T. versicolor*. The laccase enzyme reacted with the amino acid molecules of the wool fibres *via* oxidation coupling. The coloured wool fabrics presented good colour fastness and uniformity and also exhibited excellent anti-ultraviolet properties. Salat et al. (2018) produced antimicrobial cotton medical textiles by coating them with ZnO nanoparticles and gallic acid *via* a simultaneous sonochemical and laccase-catalysed process. The coated textiles kept about 60% of their antimicrobial properties after 60 washings cycles at 75 °C.

13.5.3 Pulp and Paper Industry

In Table 13.2, recent potential application of laccases to the pulp and paper industry is presented. Thus, Abd El Monssef et al. (2016) found that crude laccase from *Trichoderma harzianum*, isolated from biodeteriorated papers, was able to decolourise different fungal pigments and, thus, it could be exploited for handling fungal pigments on documentary heritage. Aslam et al. (2016) evaluated the potential for paper pulp delignification of a purified laccase from *Aspergillus flavus*. Maximum delignification was attained operating at optimal conditions (i.e. 2 h, pH 5.2 and 40 °C) and using ABTS as a redox mediator. Engel et al. (2016) showed that the lignin dissolved in the black liquor of the alkaline polyol pulping process was effectively polymerised by a commercial laccase. In addition, the structure of lignin remained mainly unchanged making possible its use in further applications.

Nathan et al. (2018) showed the potential of a partially purified laccase from the ascomycete *Fusarium equiseti*, isolated from mangrove soil, for the deinking of old newspaper waste pulp. Ortner et al. (2018) reported that laccase-polymerised lignosulfonates could be used as a novel binder in pigment-based coating formulation, thus substituting the fossil-based styrene-butadiene latex binders. Enzymatically polymerised lignosulfonates could also be interesting in size press applications for packaging papers.

13.5.4 Degradation of Pollutants

13.5.4.1 Synthetic Dyes

About 800,000 tons of dyes are produced annually worldwide, 40% of which in Europe (Hessel et al. 2007). During the dyeing processes, 2–60% of the initial used dyes is not bound to the fabric and is lost in the effluent. This generates a large amount

of dye-containing wastewater, which causes a serious environmental concern, since most dyes are mutagenic and carcinogenic (Vanhulle et al. 2008). Therefore, dye-containing wastewater must be treated prior to its discharge into the receiving water bodies. However, the existing techniques are non-efficient and/or expensive (Cooper 1995; Stephen 1995). Hence, the development of efficient and environmentally friendly technologies to remove dyes from industrial effluents efficiently and ecologically is an urgent need. In Table 13.2 some recent publications on dye removal by fungal laccases are shown. Bankeeree et al. (2016) reported the biodegradation of the recalcitrant azo dye Reactive Black 5 by crude laccase from *T. versicolor* immobilised into xylan-polyvinyl alcohol hydrogels. The dye (50 mg/L) was decolourised about 98% in 6 h at 40 °C in the first cycle and about 55% after eight successive cycles.

Chimelova and Ondrajovic (2016) showed that a purified laccase from *Ceriporiopsis subvermispora* was able to decolourise efficiently different triphenylmethane dyes with no addition of redox mediators, although their decolouration increased in the presence of redox mediators. Dai et al. (2016) studied the decolouration of the phenolic azo dye Procion Red MX-5B by a commercial laccase immobilised on Fe_3O_4/SiO_2 nanoparticles using glutaraldehyde as a coupling agent. The dye (30 mg/L) was almost totally decolourised in 1 h. In addition, after being stored at 4 °C for 5 months, the immobilised laccase was able to decolourise the dye at the same level than the freshly prepared laccase. Das et al. (2016) showed the ability of crude laccase from *P. ostreatus* grown on paddy straw and corn husk under SSF conditions to decolourise the diazo dye Congo Red. Thus, the dye (100 mg/L) was decolourised about 37% in 20 h at 35 °C. Marim et al. (2016) assessed the ability of crude laccase from *P. sanguineus* to decolourise synthetic dyes belonging to different classes. After 24 h of incubation the dyes (1 g/L) were decolourised as follows: Remazol Brilliant Blue R by 80%, Reactive Yellow 145 by 9%, Reactive Red 195 by 6% and Reactive Black 5 by 2%. Yuan et al. (2016) compared the ability of a purified laccase isoenzyme (i.e. Lac2) and the whole crude laccase extract of *Pleurotus nebrodensis* to degrade different dyes and found that the degradation rate of Lac2 was higher for most of the dyes than the crude laccase extract. Further, they showed that Lac2 effectively removed the toxicity of the triphenylmethane dye Malachite Green against bacteria and fungi.

Rani et al. (2017) investigated the decolouration of the dye Alizarin Red S (20 mg/L) by *T. versicolor* laccase free and immobilised on ZnO and MnO_2 nanoparticles. Laccase immobilised on ZnO nanoparticles led to the highest dye decolouration (95%) followed by laccase immobilised on MnO_2 nanoparticles (85%) and free laccase (49%). Sing et al. (2017) reported de decolouration by crude laccase from *Marasmius cladophyllus* of the dyes Remazol Brilliant Blue R, Orange G and Congo Red at a concentration of 200 mg/L by76%, 54% and 33%, respectively, in 19 h with no redox mediators addition.

13.5.4.2 Emerging Pollutants

The removal of emerging contaminants from wastewater is a topic of increasing interest. The ability of laccases to remove emerging pollutants such as endocrine disruptors compounds has been recently reviewed by Barrios-Estrada et al. (2018).

In Table 13.2 some recent published research on emerging pollutant removal by fungal laccases is presented. Becker et al. (2016) reported a removal higher than 50% in 24 h for 32 of the 38 antibiotics tested by a commercial laccase immobilised on a ceramic membrane bioreactor using syringaldehyde as a redox mediator. No significant removal was detected when no mediator was used. However, toxicity, assessed by the *Bacillus subtilis*growth inhibition test and the Microtox assay, increased after the LMS treatment. Chao et al. (2016) tested three redox mediators, namely, p-coumaric acid, syringaldehyde and acetosyringone, to remove carbamazepine (20 μM) by free and immobilised laccase. The best results (i.e. 60% in 96 h) were obtained by p-coumaric acid with laccase immobilised on TiO_2 nanoparticles. Further, efficient carbamazepine removal (i.e. 71% in 96 h) was also achieved in a membrane hybrid reactor with immobilised laccase. In addition, toxicity, determined by an algal viability test, was effectively removed by the LMS treatment.

Maryskova et al. (2016) investigated the ability of a commercial laccase immobilised on polyamide 6/chitosan nanofibers modified using two different spacers (bovine serum albumin and hexamethylenediamine) to remove a mixture of the two endocrine disruptor compounds (EDCs) bisphenolA (BPA) and 17α-ethinylestradiol. The two EDCs (50 μM) were efficiently removed by the immobilised laccase in three treatment cycles. Nguyen et al. (2016) showed that a commercial laccase immobilised on granular activated carbon was able to remove efficiently the micropollutants sulfamethoxazole, carbamazepine, diclofenac and BPA (each at 2.5 mg/L). Micropollutant removal was due to both adsorption on granular activated carbon and laccase action.

De Freitas et al. (2017) studied the degradation of BPA in aqueous solutions by crude laccases from *P. ostreatus* and *Pleurotus pulmonarius*. They found that 100 mg/L and 200 mg/L of BPA were removed by 100% and 85%, respectively, in 1 h by both laccases. They also found that *P. ostreatus* laccase decreased significantly the toxicity of BPA, whereas *P. pulmonarius* laccase did not. Liang et al. (2017) evaluated the water extract of soybean meal as a natural low-cost redox mediator for the degradation of sulfadimethoxine by a commercial laccase. A removal of 73.3% and 65.6% in 9 h was attained for a concentration of 1 mg/L and 10 mg/L, respectively, by the LMS using soybean meal extract as a redox mediator. In addition, the degradation rate achieved was higher than those obtained by using HBT, ABTS and p-coumaric acid as redox mediators. Taheran et al. (2017) reported the removal of the widely used antibiotic chlortetracycline from aqueous media by *T. versicolor* laccase immobilised on home-made polyacrylonitrile-biochar composite nanofibrous membrane. The immobilised laccase degraded chlortetracycline (0.2 mg/L) in continuous mode by 58.3%, 40.7% and 22.6% operating at 1, 2 and 3 $mL/h•cm^2$. Zeng et al. (2017) investigated the degradation of the herbicide isoproturon in aqueous solutions by *Trametes versicolor* laccase alone and in the presence of the redox mediator HBT. It was found that laccase alone hardly degraded the herbicide. However, in the presence of HBT, isoproturon was totally degraded in 24 h. Also, the transformation products of isoproturon after the LMS treatment showed much lower ecotoxicity to green algae than the original isoproturon.

Brugnari et al. (2018) reported that a partially purified laccase from *P. ostreatus* immobilised on MANAE-agarose was more efficient in the degradation of BPA at

high concentrations (100 mg/L) than the free laccase. In addition, the immobilised laccase retained more than 90% of its initial ability to degrade BPA after 15 cycles of reuse. García-Morales et al. (2018) studied the removal of acetaminophen (10 mg/L) and diclofenac (10 mg/L) by *Pycnoporus sanguineus* laccase immobilised on titania nanoparticles. The former was degraded by 68% in 8 h and the latter more than 90% in 2 h in aqueous solutions. Nagdhi et al. (2018) investigated the removal of carbamazepine from aqueous solutions by crude laccase from *T. versicolor* with and without the redox mediator ABTS. Operating at optimised conditions (i.e. 35 °C, pH 6, 60 U/L laccase and 18 μM ABTS) carbamazepine was degraded by 95% in 24 h. In addition, the biotransformation products showed no oestrogenicity according to the yeast oestrogen screen (YES) assay. Zhao et al. (2018) reported the efficient removal of BPA from aqueous solutions by laccase from a *P. sanguineus* expressed in *Trichoderma reesei*. Thus, BPA (25 mg/L) was degraded by 95% in 1 h using ABTS as a redox mediator.

13.5.5 Industrial Wastewater Treatment

There are very few studies reporting the treatment of real industrial wastewater by fungal laccases. In Table 13.2, the most recent ones are presented. Georgiou et al. (2016) reported the decolouration of molasse wastewater (1% v/v melanoidin) from a local industrial manufacturing yeast factory by commercial laccase immobilised on glass particles. A decolouration of 68% in 24 h at pH 4.5 and 28 °C was attained. Khalil et al. (2016) showed that a purified laccase from *Aspergillus flavus* was able to decolourise a real textile effluent in 4 days. Le et al. (2016) used commercial laccase immobilised into magnetic core-shell copper alginate beads to remove triclosan and the industrial dye Remazol Brilliant Blue R from real wastewater from a chemical factory. It was found that triclosan was removed about 90% in 8 h and Remazol Brilliant Blue R was decolourised at a range from 54.2% to 75.8% in 4 h. Antosova et al. (2017) reported the ability to decolourise a real textile industry effluent by laccases from the fungi *T. versicolor*, *M. thermophila* and *T. trogii* heterologously expressed in *S. cerevisiae*. After 4 days, the decolouration by *T. trogii* laccase was 65% and by *M. thermophila* laccase 48%. However, *T. versicolor* laccase was not able to decolourise the effluent. Vats and Mishra (2017) showed that crude laccase produced by *Cyathus bulleri* grown on wheat bran was able to decolourise and detoxify a real textile effluent by around 40% in 6 h.

13.5.6 Biosensors

Laccase-based biosensors hold great potential to be applied in the food industry, environmental monitoring and biomedical analysis. Thus, Rodriguez-Delgado et al. (2015) published an overview on laccase-based biosensors for the detection of

phenolic compounds in industrial applications. These compounds are found in natural water bodies as a result of the effluents discharged by several industries such as coal refineries, petrochemicals, pharmaceuticals, textiles, etc. Most phenolic compounds (e.g. organophosphates, PAHs, emerging pollutants) are toxic and, thus, subjected to regulation by the environmental authorities as water pollutants. Hence, their analysis, control and monitorisation are required.

The laccases used in the development of biosensors belong mainly to the genera *Aspergillus* followed by *Trametes* and *Ganoderma* (Rodriguez-Delgado et al. 2015). Bilir et al. (2016) constructed a fibre optic laccase-based biosensor for the detection of polyphenolic compounds. The constructed biosensors proved to have high reproducibility, stability and convenient measurement duration. Therefore, it has potential to be used in the food industry and in environmental monitoring to detect phenolic compounds. Palanisamy et al. (2016) reported the fabrication of a sensitive and selective laccase biosensor for the detection of catechol (detection limit 0.093 μM) using laccase immobilised on graphene-cellulose microfibres composite modified screen-printed carbon electrode for the first time. Vasilescu et al. (2016) developed a laccase-based biosensor using the electrochemical and catalytic properties of molybdenum disulphide nano-flakes and graphene quantum dots. The developed biosensor determined efficiently caffeic acid (detection limit 0.32 μM) as well as the content of total polyphenolics in samples of red wine. Verrastro et al. (2016) fabricated a laccase-based biosensor to determine phenolics using matrix-assisted laser evaporation as an innovative enzyme immobilisation technique. The developed biosensor was successfully used to determine total polyphenols in plant extracts (i.e. tea infusion, ethanolic extract from *Muscari comosum* bulbs and aqueous food supplement from black radish root and artichoke leaves).

Battista et al. (2017) reported the fabrication of a textile wearable laccase-based biosensor for the detection of L-tyrosinase in aqueous solutions (detection limit 10^{-8} M) without the use of electron mediators. Maleki et al. (2017) developed a novel laccase-based biosensor/artificial neural network integrated system to detect and measure catechol. The novel developed biosensor was successfully used to determine catechol (detection limit 0.032 μM) in real water samples (i.e. tap water and river water supplemented with catechol). Povedano et al. (2017) constructed a laccase-based biosensor by immobilising a commercial laccase on a glassy carbon electrode modified with a novel composite material consisting of reduce graphene oxide/rhodium nanoparticles. This electrochemical biosensor was successfully used to determine 17β-estradiol (detection limit 0.54 pM) in spiked real and synthetic urine samples. Vlamidis et al. (2017) built a laccase-based biosensors by immobilising a commercial laccase on a glassy carbon electrode modified with graphene oxide and multiwalled carbon nanotubes. Further, the practical applicability of the constructed biosensor was shown by determining the total polyphenols concentration in commercial fruit juice samples.

Patel et al. (2018) reported the development of amperometric laccase-based biosensors by immobilising a commercial laccase on Fe_2O_3 yolk-shell particles modified with different functional groups. The developed biosensor showed high recovery

of the phenolic compound 2,6-dimethoxyphenol from synthetic wastewater. Zheng et al. (2018) developed a laccase biosensor by immobilising a commercial laccase on modified flower-shaped yolk-shell SiO_2 nanospheres. The developed biosensor exhibited high selectivity to detect catechol (detection limit 1.6 μM) in aqueous samples.

13.5.7 Organic Synthesis

The ability of laccases to polymerise compounds makes them very useful to synthetise organic compounds in an eco-friendly manner. In Table 13.2 some recent investigations on laccase synthesis are compiled. Aljawish et al. (2016) synthesised two new compounds from the coupling between carnosine and ferulic acid using an industrial laccase as a biocatalyst. The biocatalysed reaction occurred in aqueous medium under mild conditions (i.e. pH 7.5, 30 °C). These new compounds present higher hydrophobicity, about ten-fold antioxidant properties and almost 18-fold higher anti-proliferative properties than carnosine. De Salas et al. (2016) reported the synthesis of green polyaniline (emerald salt) by using the laccase 7D5L, a high redox potential laccase produced by their group (Camarero et al. 2012; Pardo et al. 2012). The enzymatic synthesis of polyaniline is an environmentally friendly alternative to the use of harsh chemicals and extremely acid conditions. In addition, the polymer obtained (i.e. green polyaniline) displayed excellent electrochemical and electroconductive properties in water-dispersible nanofibers. Jaufurally et al. (2016) synthesised syringaresinol from sinapyl alcohol at a very high yield (93%) by using laccase from *T. versicolor* as a biocatalyst. The obtained syringaresinol showed sufficient chemical purity and good thermal and antiradical activities to be used without further purification in many polymer synthesis applications as a non-toxic alternative to the endocrine disruptor bisphenol A. Polak et al. (2016) showed the laccase-catalysed synthesis of an orange-coloured phenoxazine compound (2-amino-3-oxo-3H-phenoxazine-8-sulfonic acid) having good anti-oxidative and dyeing properties. However, no antibacterial properties were detected.

Qwebani-Ogunleye et al. (2017) used a commercial laccase to synthesise coumestans with anticancer activity. Abdel-Mohsen et al. (2017) reported the synthesis of novel pyrimidobenzothiazoles, with yields between 76 and 97%, by a laccase-catalysed reaction between unsubstituted catechol and 2-thioxopyrimidin-4(1*H*) ones using aerial O_2 as the oxidant in aqueous solution under mild reaction conditions. The synthesised compounds showed cytotoxicity against HepG2 cell line. Lim et al. (2018) reported the synthesis of low molecular weight oligomers from lignin monomers and dimers by a laccase-catalysed reaction using oxygen as an oxidant in aqueous medium. The produced oligomers exhibited notable UV blocking ability comparable to that of commercial UV blockers. Rahimi et al. (2018) synthesised a range of 1-sulfonyl-1,2,4-triazolidine-3,5-dione derivatives by the laccase-catalysed aerobic reaction of 4-substituted urazoles with sodium arylsulfinates in aqueous solution.

Rouhani et al. (2018) showed the use of a commercial laccase immobilised by covalent binding on graphene oxide-based magnetic nanoparticles as a nanobiocatalyst for the green synthesis of arylsulfonyl benzenediols from benzenediols and sodium benzenesulfinates. The immobilised laccase could be reused up to 10 times with little loss of activity. Schirmann et al. (2018) obtained dimers from 2,6-dimethoxyphenol (2,6-DMP) using laccase from *Botryosphaeria rhodina* as a biocatalyst in aqueous medium. One of the produced dimers (3,3′,5,5′-tetramethoxybiphenyl-4,4′-diol) showed application as an antioxidant for biodiesel.

13.6 Conclusion and Future Prospect

The search for environmentally friendly and sustainable methods has increased the interest in enzyme research. In this context, laccases appear as promising enzymes for the development of enzyme-based oxidation technologies due to their low substrate specificity and to the fact that they only need molecular oxygen to exert their catalytic action producing water as the only by-product. Thus, laccases have already found practical commercial applications as industrial biocatalysts in pulp bleaching (Lignozym®-process), denim finishing (DeniLite® and Zylite) and to prevent taint in cork stoppers (Suberase®). Additionally, different authors have reported very promising results in the application of laccases to different biotechnological processes. However, much research is still needed to wholly exploit the enormous potential of laccase enzymes in the development of green bioprocesses. Therefore, laccase is an old enzyme with a promising future.

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Chapter 14
Fungal Enzymes for the Textile Industry

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Abstract The wastewater discharged from the textile industry is toxic to the biological world because of the dark color and discharge of synthetic dyes. The textile industry is the largest consumer of the water for the various processes involved in dyeing and finishing and contribute to the discharge of an equal amount of wastewater or effluent into natural water bodies. It usually blocks sunlight, which hinders the life of aquatic organisms, causing the ecosystem even more problems. These discharged effluents from industry are resistant to degradation in the conventional biological treatment process. The potential of fungi has been proven for their dye degradation abilities. The main advantage to working with fungi is that they are easy to culture and can grow more quickly. The dye degradation ability of the fungi can be enhanced by the molecular genetic manipulation. Fungi are perfectly able to catabolize chlorinated and aromatic hydrocarbon-based organic pollutants, which can be mineralized by using them as an energy source.

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14.1 Introduction

Advancements in technology have facilitated and changed humans' lifestyles; these changes are the result of the degradation of the biosphere and environmental pollution that are increasing on a daily basis (Singh et al. 2017a, b; Kaur et al. 2017; Kumar et al. 2016, 2017). Owing to human negligence, an important source for the survival of the mankind—water—is at great risk. Organic and inorganic solid materials, acid and bases, and pesticides of different colors are the major contaminants present in wastewater (Ozturk et al. 2016; Singh et al. 2016a, b, c; Mishra et al. 2016; Kumar et al. 2014a, 2015a, b). Of them all, color is the most obvious undesirable property and is mainly due to the use of synthetic dyes (Gupta et al. 2005). Tannins and lignin are the coloring agents that impart color to fabrics; among complex pigments, textile dyes are predominant in all types of coloring agents (Anjaneyulu et al. 2005). The wastewater or effluent that is discharged by industry is toxic to the biological world because of its dark color; this blocks sunlight, which hinders the life of aquatic organisms and causes many problems for the ecosystem (Choi et al. 2004). Most dyestuffs presently used in industry are azo-dyes (about 10,000), constituting the largest recalcitrant category of dyes on a commercial scale (Chung and Chen 2009). The untreated or improper removal of these synthetic azo-dyes in the environment is harmful to aquatic organisms (Kumar et al. 2013, 2014b; Sharma et al. 2003).

Textile printing and dyeing parameters include several processing steps, such as pretreating the fabric, dyeing with synthetic dyes, printing, and finally the finishing processes. Pre-treatment of fabric includes the de-sizing, scouring, and washing stages (Ibrahim and Eid 2016). Before coloring the fabric, both organic and artificial dyes are rendered through various polishing procedures to obtain good-quality fabric color. Finishing processes comprise softening, cross-linking, and waterproofing, but all these steps lead to water contamination. With these processing steps, different phases of production also exist, such as yarn and fabric formation, wet processing, and textile fabrication (Hasanbeigi and Price 2012). Dyeing basically includes the application of dyestuffs under appropriate conditions to produce colored fabrics (Polak et al. 2016). In contrast, printing includes the application of dyes to a restricted area on the fabric that is selected to apply the abstract of the design. Printing is based on the same essential reactions that are also involved in dyeing (El-Shishtawy 2009). The main difference in making, dyeing, and printing distinctly mainly depends upon how the color is harnessed onto the fabric—as a solution or a thick paste (Rungruangkitkrai and Mongkholrattanasit 2012). Natural and synthetic dyes are exposed to diverse finishing methods before the fabric is actually colored to maintain the quality of the fabric color (Saini 2017).

The greatest concern of the modern world is associated with the discharge of the effluent from various finishing and textile dyeing industries. The textile industry is the largest consumer of water for the various processes involved in dyeing, and finishing also contributes to the discharge of an equal amount of wastewater or effluent into natural water bodies (Mondal et al. 2017). Residues remain from reactive dyes, and complex components such as aerosols, organic and inorganic impurities cause

the coloration of the wastewater discharged from the textile dyeing unit, which has high values of chemical oxygen demand (COD) and biological oxygen demand (BOD), and also additional materials that are hard to degrade (Babu et al. 2000).

The residues of synthetic dyes are discharged into natural streams that pass by effluent treatment plants or are released directly into the water, causing severe damage and contamination (Joshi et al. 2004). The release of dye effluents in such a way into natural bodies of water is totally inadmissible, because of their hue, and owing to the presence of breakdown products of dyes, which are toxic and carcinogenic (Pereira and Alves 2012). Technology chiefly based on the bioremediation principle has been tested as a suitable and cost-effective methodology to counter textile dye pollution. The ability or power of microorganisms to decolorize and metabolize dyestuffs is used to treat the environment contaminated by textile dyes and their breakdown products (Rani et al. 2014).

A variety of the microorganisms studied so far, but mainly the potential of bacteria, has been revised because of their ability to degrade dye. In the context of bioremediation, numerous assessments have been carried out of bacteria that have the ability to catabolize the organic pollutants (Ma et al. 2005). The main advantage of working with bacteria is that they are easy to culture and they can grow more quickly than other microbes. The ability of the bacteria to degrade dyes can be easily enhanced by molecular genetic manipulation. Bacteria are perfectly able to catabolize chlorinated and aromatic hydrocarbon-based organic pollutants, which can be mineralized by using them as an energy source (carbon source) (Jain et al. 2005). Many reports have demonstrated bacteria that degrade and mineralize different azo-based dyes at a faster pace. The different bacterial groups under traditional aerobic, anaerobic, and under extreme oxygen-deficient conditions execute azo dye reduction for decolorization. The chemical reaction involved during the reduction of the azo dyes starts with the breaking of the azo bonds (-N=N-) under an anaerobic environment by the enzyme azoreductase , which forms the colorless solution of aromatic amines that are probably harmful (Chang and Kuo 2000; van der Zee and Villaverde 2005). Seshadri et al. (1994) reported that metabolites formed after the dye reduction can be further catabolized by either the aerobic or the anaerobic process. The intermediate products synthesized during dye decolorization can also be reduced by other enzymes, such as hydroxylase and oxygenase, which are also produced by bacteria (Elisengela et al. 2009; Wang et al. 2009). Several bacteria that possess the ability to degrade the azo dye to the colorless amines have been reported. Oller et al. (2011) assessed the behavior of aerobic bacteria that were able to propagate even when the azo compounds were present. The intermediate sulfonated amines formed in this process may be aerobically degraded. Gram-positive bacterial strains, including *Clostridium perfringens*, *Bacillus cereus*, *Brevibacillus* sp., and *Paenibacillus azoreductase*, were found to be efficiently decolorizing various structurally different textile azo dyes. Similarly, Gram-negative bacterial strains, including *Klebsiella pneumoniae*, *Proteus mirabilis*, *Pseudomonas putida*, *Citrobacter* sp., and *Escherichia coli*, exhibited promising decolorizing efficacy with regard to various dyes (Franciscon et al. 2012; Singh and Singh 2017).

14.2 Enzymatic Degradation of Dyes

Enzymes can be defined as the molecules that facilitate the sequential breakdown and degradation of the different dyes. The initial step involves the dissociation of the electrophilic bonds of azo that result in the instant decolorization of azo dye. The enzyme azo-reductase brings about the cleavage of azo linkages in organic compounds that contain azo bonds, resulting in the production of aromatic amines. Many bacterial species have been discovered to comprise unspecific cytoplasmic enzymes that act like azo-reductase (Lade et al. 2015; Popli and Patel 2015). Chang et al. (2001) describes the class of the enzyme named Azo-reductases that involves the catalytic reduction reaction that results in the breakdown of the bond of the azo group (-N=N-) to the synthesis of aromatic amines that are colorless. Several researchers have reported the application of bacterial cytoplasmic azo-reductase in environmental biotechnology (Moutaouakkil et al. 2003; Maier et al. 2004; Ramalho et al. 2004). Azo-reductase can be classified on the basis of the primary amino acid level, which is found to be difficult; hence, recently, they have been classified based on the analysis of secondary and tertiary amino acids (Abraham and John 2007). The reaction of the breakdown of the azo bond by azo-reductase is shown in Fig. 14.1.

The phenol-oxidases, namely laccases, have great potential to degrade many aromatic compounds (Levasseur et al. 2008). These laccase enzymes execute the degradation of complex polyaromatic polymers, called lignins. Laccases (EC 1.10.3.2) belong to the class of oxidoreductases, also multicopper oxidases, which

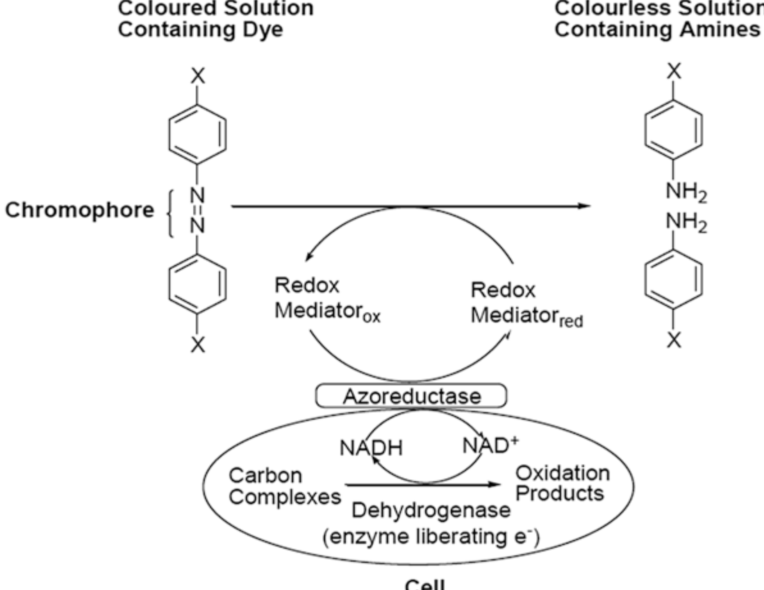

Fig. 14.1 Proposed mechanism for the reduction of azo dyes by azo-reductase (Keck et al. 1997)

have the ability to oxidize the phenols, polyphenols, and aniline by removing one electron (Kudanga et al. 2011). Manganese peroxidases are enzymes that are members of the class of oxidoreductases. Abadulla et al. (2000) stated that manganese peroxidases attack phenolic compounds through the intermediary redox reaction with the help of Mn^{2+}/ Mn^{3+} ions, whereas lignin peroxidase attacks the nonphenolic methoxy substituted lignin subunits, which behave as substrates.

Laccase enzymes employ the mechanism of free radicals that are nonspecific in nature to execute the degradation of the azo dyes without forming toxic aromatic amines (Jadhav and Phugare 2012). Kalme et al. (2009) testified optimal results of the laccase enzyme recovered from *Pseudomonas desmolyticum* strain NCIM 2112, which showed 100% degradation of dyes such as Direct Blue-6, Red HE7B, and Green HE4B. The reaction mechanism catalyzed by laccase enzymes is illustrated in Fig. 14.2.

Fig. 14.2 Reaction catalyzed by laccases

Enzymes are primarily globular proteins containing long linear chains of amino acids that fold to form a three-dimensional structure. Each and every specific amino acid sequence produces an entirely different structure with various features. This makeup of amino acid sequences renders one enzyme different from another, in both structure and function; hence, they are easily identifiable (Alberts et al. 2002). It has been keenly observed that enzymes take part in various steps involved in different metabolic pathways in a healthy cell and it has been estimated that enzymes catalyze more than 5000 biochemical reactions of variable types (Vastrik et al. 2007).

Enzymes are mostly proteins, but as an exception, a few RNA molecules are also found to have enzymatic characterization and functions. One of the most common feature of RNA enzyme is the absence of enzymatic catalysis, which contains more than half of the biological metabolic pathways and chemical reactions that occur in a cell which are extremely slow and would not occur under mild temperatures and pressure conditions that are compatible with life (Cooper 2000). Enzymes speed up the rate of such reactions by numerous -fold; hence it can be said that reactions that would take several years to complete in the absence of enzymatic catalysis can occur in a few seconds or even a fraction of seconds if catalyzed by a significant enzyme. Because of their catalytic activity, enzymes are also known to be "biological catalysts" as they speed up or increase the reaction rate of any metabolic and chemical reactions by lowering their activation energy. The molecule upon which the enzyme acts is primarily known as a substrate, which is converted into a product. Active site and substrate specificity are responsible for product formation. Product formation takes place in two ways, i.e., either one substrate is broken down into a number of products or two substrates come together to form one larger molecule (Robinson 2015).

The part of the protein molecule where the substrates bind is primarily known as the active site. The binding of the substrate onto the active site happens because of an induced fit mechanism, but it was once thought that the substrate binds to the enzyme in a lock and key manner; hence, the enzyme binding concept, the lock and key model came about, which over a period of time was superseded by the induced fit mechanism, where the enzyme undergoes a set of modifications to fit the substrate to its active site and also to make the interaction between the two stronger, resulting in an enzyme–substrate complex (Linenberger and Bretz 2015). After that, it goes through a series of reactions to yield a product. An extremely significant feature of an enzyme is that it does not remain bound to the reacting molecule and they are not altered by the reaction they catalyze, which basically implies that at the end of the reaction, the enzyme that acts as a catalyst releases the products and gets ready to take part in another reaction to catalyze it (Robinson 2015). This key role played by enzymes makes them highly crucial macromolecular biological catalysts. Almost all the metabolic processes in the living cell require enzyme catalysis to accelerate their processing rate and because of these crucial factors, enzymes are gaining widespread importance in the industrial sector (Gurung et al. 2013).

Various enzymes have been discovered that have great importance and are efficiently used in industry. One of the industries utilizing enzymes in production and other processes is the textile industry. It is analyzed that textile production and pro-

cessing benefit highly with regard to product quantity, quality, and environmental impacts from the use of enzymes (Singh et al. 2016a, b, c). There are approximately 7000 enzymes known, of which 75 are commonly used in the textile industry including: hydrolases, which encompass amylases, cellulases, proteases, pectinases, lipases, and esterases (Ahuja et al. 2004).

14.3 Salient Features of Enzymes Used in Textiles

Enzymes are very specific for the type of reaction without any side effect. They consume less energy and small amounts can be used for a safe and noncorrosive catalyzing reaction. Under unfavorable conditions, enzymes alter their physical configurations, which result in denaturation and activity loss. Changes in pH and temperature affect the activity of most of the enzymes. Use of ionic surfactants limits their activity, whereas non-ionic wetting agents with an appropriate cloud point increase their working efficiency. Enzymes are sensitive to heavy metal concentration and it is mandatory to exercise extreme caution at the time of its application. They are highly biodegradable and result in reduced loads on the effluent treatment plant. The use of enzymes in the textile industry decreases the amount of chemicals discharged into wastewater that generate a safe environment for textile workers. This results in superior fabric quality, an increase in the life of the original fabric, and leads to longer garment life. It decreases chemical usage, decreases water utilization, and reduces energy usage. Overall, enzyme use in the textile industry is environmentally friendly in nature.

14.4 Properties of Enzymes Used in Textiles

Enzymes are considered to be bio-degradable and eco-friendly as they degrade toxic chemicals to a nontoxic from within the process so that these chemicals can be disposed of in nature without harming nature. They serve as a better alternative to chemicals. Formerly, the textile industry used various toxic chemicals for different processes, but these have now been replaced by enzymes. This has made the treatment of the wastewater cost-effective. Generally, the role of the enzyme is to catalyze the reaction without amending its natural state. In chemistry, a catalyst enzyme is stated to be a substance that increases the rate of reaction by lowering the activation energy. Enzymes are substrate-specific and catalyze a specific substrate. Enzymes are considerably dependent on the temperature and pH of the reaction, which decides their optimal activity (Table 14.1). These ideal conditions make the regulation of enzymes easier. Enzymes regains its orginal form once product is released and they can be reused again. Moreover, use of enzymes in the textile industry has reduced water consumption by 19,000 liters per ton for the bleaching process.

Table 14.1 Enzyme treatments and conditions employed in textile industry processes

Types of enzyme	Enzyme conc. (%)	Temperature (°C)	pH	Industrial processes	References
Cellulases	1–5	40–60	4.8	Biofinishing, biopolishing	Ali et al. (2012)
Amylases	0.5–1.0	40–50	6.5–8.0	Sizing agents for denim	Colomera and Kuilderd (2015)
Proteases	0.8	40–60	6–8	Diffusion of the dye into the fibers	Periolatto et al. (2011)
Lipases	0.5–4.0	40	8	To enhance its dyeability with a basic dye	El-Shemy et al. (2016)
Pectinases	0.7–0.8	40	5.5–6.5	In textile processing and bioscouring of cotton fibers, desizing	Mojsov (2012a)
Catalases	0.04	60	ND	Elimination of hydrogen peroxide residues after bleaching of cotton fabrics	Amorim et al. (2002)
Peroxidases	4.5–5.0	50	5	Used as an enzymatic rinse process after reactive dyeing, the oxidative splitting of hydrolyzed reactive dyes on the fiber, and biobleaching of important industrial dyes	Osuji et al. (2014)
Ligninases	ND	30	3.7–4.9	Decolorization of eight synthetic dyes including azo, anthraquinone, metal complex, and indigo applications in a large number of fields, including the chemical, fuel, food, agricultural, paper, textile, cosmetic industries	Young and Yu (1997)
Collagenases	0.1	60	7	Biocatalysts in the exhaustion of dyes	Kanth et al. (2008)
Esterases	ND	ND	6	Partial hydrolysis of synthetic fiber surfaces, improving their hydrophilicity and aiding further finishing steps	Araujo et al. (2008)
Nitrilases	ND	ND	7.4	In the development of polyacrylonitrile preparation for better coloration in textile processing	Robinson and Hook (1964)

14.5 Production of Enzymes: Searching for Efficient Production Systems

Different biological sources such as animals, microbes, and plants serve as the commercial source for enzyme production. However, these naturally occurring enzymes are not readily available to meet the need of industry. To overcome this,

recombination techniques enable us to exponentially increase enzyme production and identify enzymes of industrial importance (Adrio and Demain 2014; Li et al. 2012; Sahay et al. 2017; Suman et al. 2015). To express the heterologous proteins, different hosts such as *E. coli*, *S. cerevisiae*, *P. pastoris*, filamentous fungi, and mammalian cell lines have been developed (Nierstrasz and Cavaco-Paulo 2010). Several advantages, e.g., rapid and high growth on the cheap carbon source, easy scaling-up, being genetically well-characterized, large cloning vectors, and mutant strains make *E. coli* arguably the most effective expression vector (Rosano and Ceccarelli 2014). Still, sometimes it is found to be unsuitable as it is devoid of auxiliary biochemical pathways, vital for certain phenotypic expression, which does not guarantee that the recombinant product will able to accumulate in *E.coli* in a full-length and active form (Araujo et al. 2008). To overcome this, the genes are cloned into similar species from which the genes were obtained. If a heterologous protein needs post-translational modification (PTM), which are difficult to express in a prokaryotic system, yeast are used as an alternative to synthesizing eukaryotic proteins (Merlin et al. 2014). Within the yeast species, *Pichia pastoris* serves the purpose, as its growth rate is high, and it can be easily scaled-up by the fermentation procedure without affecting the yield. Moreover, secretion of recombinant protein along with endogenous protein makes PTM such as glycosylation and proteolytic processing, and the purification of enzyme more convenient (Ahmad et al. 2014; Shen et al. 2016). Furthermore, the advance in genetic engineering enables us to incorporate more copies of the expression cassette with recombinant DNA into the expression vector, eliminates the problem within the expression plasmid, making the yeast the vector of choice for various industrial processes (Nierstrasz and Cavaco-Paulo 2010).

14.6 Role Played by Enzymes in the Textile Industry

As discussed, there are various enzymes that are of importance in the textile industry. Out of 7000, a total of 75 enzymes are used effectively and efficiently for carrying out textile processing and production (Gessesse et al. 2011; Nigam 2013). There are various fungal enzymes that have proved their exclusive role in the textile industry (Fig. 14.3). The detailed characteristics, attributes, and roles of the some of the enzymes are described in the sections below.

14.6.1 Amylases

Amylases are enzymes that hydrolyzes starch into small polymers of glucose, including dextrins (Windish and Mhatre 1965). These are further divided on the basis of the type of sugar synthesized, i.e., α- and β-amylase. Mostly, α-amylases are synthesized by the various bacteria, fungi, and yeast, but bacteria and filamentous fungi are mostly used in industry, and in the agriculture and allied sectors in

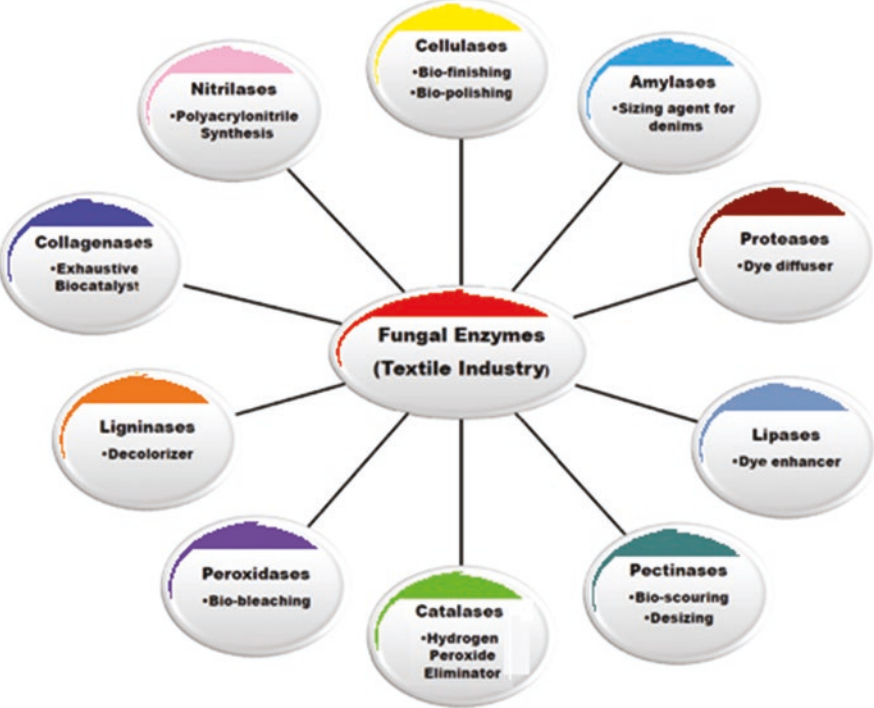

Fig. 14.3 Fungal enzymes and their important role in the textile industry

different processes (Singh et al. 2016a, b, c; Yadav et al. 2016a, b; Sivaramakrishnan et al. 2006). Microbial α-amylase generally falls within the range 50–60 kDa, with the exception of *Bacillus caldolyticus* and *Chloroflexus aurantiacus*, which produces 10 kDa and 210 kDa α-amylase respectively (Grootegoed et al. 1973; Ratanakhanokchai et al. 1992). It has been found that most amylase-producing bacteria and fungi are stable within the pH range of 4–11. In comparison with the extremely alkalophilic *Bacillus sp*. and the alkalophilic *Sulfolobus acidocaldarius*, *S. acidocaldarius* is found to be stable at pH 3 (Krishnan and Chandra 1983; Lee et al. 1994; Schwermann et al. 1994; Kim et al. 1995). The optimal temperature for α-amylase activity is largely dependent on the growth temperature of the microbe synthesizing the enzyme (Vihinen and Mantsala 1989). In 1985, Chary and Reddy reported that a temperature of 25–30 °C is optimal for α-amylase-producing *Fusarium oxysporum*, whereas other reports provided evidence that α-amylase-producing *Pyrococcus furiosus* and *Pyrococcus woesei* were stable and active at 100 and 130 °C respectively (Laderman et al. 1993; Koch et al. 1991). It has been reported that in some cases, the addition of Ca^{2+} ions enhances the thermostability of microbes (Vihinen and Mantsala 1989), whereas severe inhibition is observed when they are exposed to ethylenediaminetetraacetic acid, egtazic acid, sulfhydryl reagents, and heavy metals (Mar et al. 2003; Tripathi et al. 2007). Mostly, α-amylases

obtained from microbes have specificity for starch followed by amylopectin, cyclodextrin, glycogen, and maltotriose (Vihinen and Mantsala 1989).

Cotton blends have warp threads that are coated by an adhesive named “size,” which greases and prevents the abrasion of yarn threads during the weaving process. However, various materials have been used to size the fabrics, but starch and its derivatives are most commonly used, as they have a vivid film-forming ability, easy availability, and low cost (Feitkenhauer et al. 2003). For the dyeing and finishing of the fabric, it was necessary to remove sizing agents, which were previously treated with acid, base, and an oxidizing agent at a high temperature before the discovery of amylases. The chemical treatment given to fabric was found to be ineffective, as it caused imperfect dyeing, degrading the cotton fiber and its natural soft feel. Currently, amylases are commercially used for the resizing process, as they do not affect the fabric because of their high specificity and efficiency (Etters and Annis 1998; Cegarra 1996). Amylases randomly cleave the starch into dextrins (water soluble in nature), which are easily removed during washing. As a result, the discharge of waste chemicals into the environment is reduced (Feitkenhauer et al. 2003).

14.6.2 Cellulase

The enzyme cellulase is hydrolytic in nature and is used as a catalyst that breaks down cellulose into oligosaccharides and then into glucose. The multi-component enzyme system comprises of at least three kinds of cellulases that major function is to shown cellulase activity (Horn et al. 2012). There are two significant enzymes that cleave the bonds along the length of the cellulose chain in the middle of the amorphous region: endo-glucanases and endo-cellulases. Apart from these two enzymes, there are enzymes, e.g., cellobiohydrolases or exocellulases, which play a specific role, to produce cellobiose, and are of utmost importance (Zhang and Zhang 2013). It was also observed that a mixture of one type of enzyme or a number of types of enzymes gives better results as they are more efficient at activity and processing compared with the sum total of the activities of all the individual enzymes. One such example is the synergistic characteristic shown by cellobiohydrolases with each other or with endo-glucanases (Horn et al. 2012; Zhang and Zhang 2013). Cellulase enzymes have been reported to be produced by diverse groups of microbes, including archaea, bacteria, fungi, and cyanobacteria (Horn et al. 2012; Saxena et al. 2016; Verma et al. 2017; Yadav et al. 2017a, b, 2018).

The optimal temperature requirement of cellulases is 30–60 °C and the optimal pH requirement of this industrially important enzyme is 4.5–5.5; hence, they are classified as acid stable (Sadhu and Maiti 2013). The large-scale use of cellulase started during the late 1980s, to stain denim. It is widely used in textile wet processing and works as a modifier for the fiber surface. Since then, it has gained widespread importance in washing denim to obtain a stone-washed appearance, without using stones (Margado et al. 2000). Cellulase has various effects on man-made

fabrics made of cellulose, for example, lyocell, which is also known as TENCEL, is affected by cellulase activity resulting in the alteration of drapeability, and its activity also results in removal of surface fuzz (Sukharnikov et al. 2011). The same effect is shown by cellulase on viscose. Also, cellulase is seen to reduce the capability of viscose to pill and reduces the tendency of lyocell to fibrillate. It is found that when cellulase is pretreated, the uptake of natural dyes such as chlorophyll and carmine are increased greatly by cellulase, without affecting fastness Not only does it give denim a stone-washed appearance, it is also used to confer to fabrics such as cotton, linen, and knits an improved appearance and handling. The main enzyme responsible for this activity is cellosoft L (Kumar et al. 1994). The best and most advantageous uses of cellulase are: ageing of fabric surfaces; the stone-washed look of denim clothes or outfits, and cleaning and renewing of fabric surfaces that are destroyed because of micro-fibrils, fuzz, and loose fibers.

To obtain such useful results, each kind of enzyme has a different composition. For example, the composition of endoglucanases (EG) or EG-rich preparation is used effectively for the ageing and defibrillation of the fiber surface, whereas to obtain the best cleaning and de-pilling effects, complete cellulase systems are used (Andreaus et al. 2014). One observation taken into consideration was that when cotton fabrics were treated with a cellulase mixture of a distinct composition, followed by washing in a process where mechanical agitation takes place, the fiber surface did not show any change after cellulase treatment. However, when it was washed, the surface showed different properties that were directly linked to the quality of the enzyme used (Cheung et al. 2013). In addition to the bio-stoning process, both natural and manmade cellulosic fibers can be improvised and their quality can be increased by a process called bio-polishing, which involves the activity of cellulase, and its main advantage is preventing pilling (Andreaus et al. 2014). Pilling apparently consists of a fuzz ball that when formed on a garment deteriorates its quality by making it unattractive with knotty fabric pilling. However, when garments are made to undergo bio-polishing, the fabric tends to show much less pilling formation. Removal of pilling or balls of fuzz has various other advantages, giving the garment a softer and smoother fabric texture and excellent color brightness. It has also been observed and analyzed that the softness-enhancing effects of cellulase provide waterproof and nongreasy qualities (Namrata 2012).

14.6.3 Catalases

Catalases, which are more appropriately known as hydroperoxides, play a role in catalyzing the degradation of H_2O_2 to H_2O and O_2. They are produced by numerous microorganisms, including bacteria and fungi (Zámocký et al. 2012). Their optimal temperature requirement ranges from 20 to 50 °C and works best and efficiently at a neutral pH. Catalases obtained from animals are basically cheaper; therefore, the production of catalase from microbes would only be economically feasible and

advantageous when cheap technology and recombinant strains are used (Yumoto et al. 2000).

After desizing and scouring, bleaching of H_2O_2 can take place, but before dyeing. Catalases are also used to decompose an excess of H_2O_2 and apparently, this obviates the requirement for a reducing agent and decreases the need for rinsing water, resulting in lower polluted wastewater and a lower consumption of water (Ul Aleem 2013). By introducing immobilized enzymes, the cost of enzymes for degrading hydrogen peroxide in bleaching effluents could be easily reduced. This also allows the recovery of enzymes and the reuse of treated bleaching effluents for dyeing (Araujo et al. 2008).

14.6.4 Laccases

Laccases are a multi-copper enzyme, are extracellular, and use molecular oxygen to oxidize phenols, a variable number of aromatic and non-aromatic compounds by a radical catalyzed reaction mechanism (Jeon et al. 2012). Laccases are found in plants, insects, and bacteria, but are abundant in fungi. More than 60 fungal strains show laccase activity. The size of a typical fungal laccase is approximately 60–70 kDa and requires optimal environmental conditions for proper functioning (Brijwani et al. 2010). The required optimal temperature is 70 °C, whereas some require 35 °C for efficient activity. The favorable pH conditions of laccase range in acidic value. One of the major features peculiar to laccase enzymes is that they lack substrate specificity, which implies that they react over a broad range of substrates (Shekher et al. 2011).

Bleaching is used to create a variety of shades on denim outfits. Basically, bleaching powder, chemically known as sodium hypochlorite, is used to bleach denim garments. By controlling, transmuting, and manipulating different washes, for example, ice wash, a Bata wash is created. However, this process has a serious drawback, as chlorine-based bleaches are used, which are not environmentally friendly and also create a hazardous and unsafe working environment (Haq et al. 2015). Laccase is an enzyme that is known to decolorize indigo. In addition, it is a very important enzyme for the treating and finishing of denim fabrics. It is widely used in textile processing because of its significant feature, i.e., its ability to decolorize textile effluents. Because of its ability to degrade dyes of various structures, including synthetic dyes, laccase is an environmentally friendly agent for treating dye wastewater (Doshi and Shelke 2001). The whitening of cotton by the oxidation of flavonoids is done with the help of laccase. It was found that the combination or substitution of chemical bleaching with enzymatic bleaching results in less fiber damage and saves water (Mojsov 2012b). Laccases of the organism *Trametes hirsuta* are responsible for the oxidation of the flavonoids morin, luteolin, rutin, and quercetin. There is various evidence that the pre-treatment of cotton with laccases obtained from *T. hirsuta* results in an increase in whiteness. Ultrasound was also

used to increase the efficiency and activity of enzymatic bleaching. Apart from this, it was observed that when low-intensity ultrasound was used, there was improved diffusion of the enzyme from the liquid phase to the fiber surface (Kim et al. 2008; Araujo et al. 2008).

14.6.5 Trans-glutaminases

Trans-glutaminases are a group of thiol enzymes that have the significant feature of catalyzing the post-translational modification of protein, primarily by protein-to-protein cross-linking; this is also done through the covalent conjugation of polyamines, lipid esterification or the deamidation of glutamine residues (Lorand and Graham 2003). This enzyme is broadly distributed among bacteria, plants, and animals. From the bacterium *Streptomyces mobaraensis* , the first microbial transglutaminase was obtained. However, it is secreted as a zymogen, which is efficiently processed by two endogenous enzymes to obtain a yield of mature form. It is a monomeric protein with a molecular weight of 38 kDa, containing a single catalytic cysteine residue with an isoelectric point of 9 (Brown et al. 2008). The optimal environmental condition required by transglutaminase is a temperature above 55 °C. It maintains full activity for 10 min at 40 °C, but loses efficient activity within a few minutes at 70 °C. The optimal pH required by the enzyme transglutaminase ranges from 5 to 8, but there are cases where it is found to show some activity at pH 4 or 9; hence, it can be concluded that trans-glutaminase works over a wide range of pH values; also, it was found to be working at 10 °C and seen to retain some activity at a near freezing temperature range (Jaros et al. 2006). Microbial transglutaminase possesses many other features, for instance, it does not need calcium for to be active (Porta et al. 2011).

14.6.6 Pectinases

Pectin and pectic substances are the polysaccharides that are found in the middle lamella and cell wall of the plants. Pectinases are enzymes that degrade these pectic substances. This enzyme is chiefly synthesized by plant pathogens (bacteria and fungi) and saprophytes to degrade the cell wall of plants (Pedrolli et al. 2009; Yadav et al. 2018). The pectinase is further divided into three main groups, i.e., pectin esterases (PEs), polygalacturonases (PGs), and polygalacturonate lyases (PGLs). Bacteria and fungi produce the pectin esterases that catalyze the hydrolysis of pectin methyl esters to form pectic acid on plants such as banana, lemon, orange, and tomato. It usually acts on the methyl ester site of galacturonate present next to non-esterified galacturonate (Muthu 2014). The molecular weight of

pectinesterase obtained from microbes and plants falls within the range 30–50 kDa. Generally, the optimal temperature is found to be 40–60 °C and pH is found to be within the range 4–7 for PEs with the exception of *Erwinia*, which shows the optimal activity in the alkaline region (Kohli et al. 2015). PG is the enzyme that hydrolyzes the α-1,4 glycosidic linkages via exo- and endo-splitting mechanisms in pectin (Palanivelu 2006). Endo PGs are most commonly obtained from bacteria, fungi, and yeast, and have a molecular weight of 3080 kDa. This enzyme is found to be active within an acidic range, i.e., 2.5–6, and within a temperature range of 30–50 °C, although exo PGs have been reported to be synthesized by plants such as apple, carrot, and peach, and *Aspergillus niger* and *Erwinia* sp. This enzyme is found to have a molecular weight within the range of 30–50 kDa (Araujo et al. 2008). PGLs are enzymes that cleave the pectin chain through β-elimination, which leads to the formation of a double bond between C_4 and C_5 present at the reducing end and liberates CO_2. The endo PGL cleaves arbitrarily, whereas exo PGL cleaves at the end of polygalacturonate to yield unsaturated galacturonic acid (Hoondal et al. 2002). The molecular weight of the PGLs falls within the range 30–50 kDa, with optimal activity within the pH range 8–10. However, PGLs obtained from *Erwinia* and *Bacillus licheniformis* are found to be active at pH 6 and 10 respectively. The enzyme PGL is typically found to be active within a range 30–40 °C, whereas PGL obtained from thermophiles are reported to have an optimal temperature within the range 50–75 °C. The pectate lyases have been extensively explored for bioscouring (Tierny et al. 1994).

The raw cotton comprises different noncellulosic impurities such as waxes, hemicellulose, and minerals salts, that accumulate in the cell wall and cuticle of the fibers. These impurities contribute to the hydrophobic nature of raw cotton, which interferes with chemical processing, e.g., the dyeing and finishing of cotton (Sawada et al. 1998). Thus, before the dyeing of the cotton yarn, pre-treatment is required to remove the materials that inhibit the binding of the dye. This process is known as scouring, which enhances the wettability of the fabric, and for which sodium hydroxide is used (Araujo et al. 2008). Although these chemicals have been known to reduce the strength of cellulose, the weight of the fabric is also reduced. Additionally, this treatment increases COD, BOD, and the salt content of wastewater generated after the treatment. Bioscouring overcomes the problem associated with chemicals. During processing by the enzyme, the cellulose remains intact, which prevents the loss of strength and weight of the material (Duran and Duran 2000). Bioscouring has various advantages over traditional scouring. This process is generally performed at a neutral pH; as a result the total water consumption is reduced to a large extent compared with the traditional procedure, and the softness of the cotton fiber is maintained (Muthu 2014). Different enzymes such as cellulases, cutinases, lipases, and pectinases have been studied individually and in combination for the bioscouring of cotton, but pectinases are found to be highly effective (Karapinar and Sariisik 2004).

14.6.7 Nitrilases and Nitrile Hydratases

Nitrilase was previously known as a nitrile-hydrolyzing enzyme, which converts the indole 3-acetonitrile to indole 3-acetic acid. The superfamily nitrilase, formed on the basis of the structure and analysis of amino acid sequences, comprises 13 branches. Only one member of the family is known to exhibit nitrilase activity, whereas the remaining members are involved in either amide condensation or amidase activity. All the superfamily members share the conserved triad of cysteine, lysine, and glutamate with a large a-b-b-a structure. Nitrilases are rarely found in nature (Duca et al. 2014). Various genera such as *Klebsiella*, *Nocardia*, *Pseudomonas*, and *Rhodococcus* have been reported to use nitrile as their sole carbon and nitrogen source (Gong et al. 2012). Advances in the field of biotechnology have enabled the isolation of the bacteria and fungi, which have the ability to hydrolyze nitrile. Commonly, isolated nitrilases possess a single polypeptide chain with a molecular weight of 3045 kDa, which aggregates to synthesize a holoenzyme under various conditions (Singh et al. 2006). The predominant form of this enzyme is the large aggregate of 626 subunits. Generally, enzyme activation depends on the substrate, although the elevated level of temperature, organic solvents, salt, pH or sometimes the enzyme itself initiates subunit aggregation (Nagasawa et al. 2000). Nitrile hydratase (NHase) is the chief enzyme involved in the enzymatic pathway responsible for the conversion of nitriles to amides, which further transforms to the corresponding acid via amidases (Duca et al. 2014). NHase has been isolated from microbes such as *Rhodococcus erythropolis*, *Agrobacterium tumefaciens*; the characterization and purification of the enzyme have also been achieved. NHases consist of two subunits, i.e., a and b, which can be complexed in different ways (Brandão et al. 2003; Okamoto and Eltis 2007; Kamble et al. 2013). These enzymes are also stated to be metalloenzymes as they may possess cobalt or iron.

Polyacrylonitrile (PAN) fibers hold 10% of the global market for synthetic fibers because of its properties such as resistance to chemicals, high elasticity, and aesthetic properties similar to natural fibers (Mikolajczyk et al. 2009). However, the hydrophobic nature of the fiber is hindered during the process of dyeing and finishing. On chemical treatment, hydrolysis causes the yellowing of the fiber. Therefore, enzymes are considered to be an effective alternative to hydrolyzing PAN (Martinkova and Křen 2002). Nitrile hydratase and amidase obtained from *A. tumefaciens* and *Rhodococcus rhodochrous* can be used for the surface modification of PAN (Prasad and Bhalla 2010). Thus, treatment with enzymes increases the hydrophilic nature of the fiber, which makes it ready for dyeing. Similar work has been reported in which PAN was treated with nitrile hydratase obtained from *Arthrobacter* sp., *Brevibacterium imperiale*, and *Corynebacterium nitrilophilus*, which caused an increase in the number of amide groups on the surface of PAN, contributing to properties such as dyeability and hydrophilicity (Gong et al. 2012). The hydrolysis of nitrile groups persisting on the PAN surface was determined by measuring the liberation content of NH^{3+} and by assessing the intensity of basic dye on fabric treated with enzymes (Fischer-Colbrie et al. 2007). Further, the addition of 4% N,N-

dimethylacetamide and 1 M sorbitol to treatment media increases the catalytic activity of the enzyme. Still, there is no industrial application; however, the research revealed that the enzymatic treatment of PAN enhances the quality of fibers, saves energy, and controls pollution (Bhatia 2017).

14.7 Conclusion and Future Prospects

Discharge from textile industries can be characterized on the basis of composition that comprises nondecomposable artificial dyes with different kinds of toxic material. The difference in the effluent discharged from the textile industry is due to the process followed for fabric pre-treatment, which contributes to the involvement of the various materials and chemicals as per the requirement of the particular industry, as their capability is dependent on different physical properties such as concentration and class of dye, pH, salinity, and production of the end product, which can be toxic**.** Moreover, promising results were shown on the utilization of the redox mediators/thermophilic treatment to catalyze the decolorization process in the bioreactor, whereas the immobilization of redox mediators in the bioreactor and their recovery during the down-stream process is still a global challenge. Furthermore, much future work is needed to isolate novel microorganisms capable of the effective degradation of a wide range of textile dyes and to create a contamination-free environment. This approach creates the potential to remediate environments polluted by textile azo dyes.

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Chapter 15
Marine Fungal White Biotechnology: An Ecological and Industrial Perspective

Anjana K. Vala, Bhumi K. Sachaniya, and Bharti P. Dave

Abstract Fungi with their matchless characteristics ranging from greater growth capacity to capability to produce a number of enzymes, etc. have gained attention in the field of biotechnology. Fungi from marine environment, owing to the ability to grow under diverse extreme conditions like high salinity and pH, could prove even better for their white biotechnological applications. Marine-derived fungi have been observed to produce several white biotechnologically important products; however, despite their noteworthy potential, they have not been explored much for their commercial applications. In this chapter, some of the ecologically and industrially relevant potentials of fungi from marine environments have been discussed.

15.1 Introduction

White biotechnology (or industrial biotechnology) involves use of living cells and/or their enzymes to produce products with less energy requirement, less waste generation, and easy degradability, at times performing even better than the products produced with conventional chemical processes. At times, purity of products is an added advantage of white biotechnology. Approximately 5% of the total chemical market is contributed by white biotechnology-based products. The biotechnological feedstock will come to be 75% of the total chemical feedstock by 2050 (Lee and Jang 2006). Marine environment is a reservoir of biodiversity. A range of ecologically relevant tasks are being performed by marine microbial communities. Besides carrying out

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environmentally important reactions, marine microbes, especially fungi from marine habitats, are reservoir of commercially relevant enzymes (Bonugli-Santos et al. 2015).

Fungi from marine habitats were first described by Duriers and Montagene (1846–1850) around the middle of the nineteenth century in France (Verma 2011). Mainly marine fungi consist of *Ascomycota* and a few *Basidiomycota* and anamorphic fungi. Based on their biogeochemical distribution, these biota can be categorized as temperate, tropical, subtropical, and cosmopolitan species. As reported by Hyde et al. (2000), marine fungi are not a taxonomic group, but they form an ecological group. Mycobiota from marine habitats have been categorized as (a) obligate marine fungi, growing and sporulating only in a marine or estuarine (brackish water) habitat, and (b) facultative marine fungi, having freshwater or terrestrial origin and capability to grow and possibly sporulate also in marine environment (Kohlmeyer and Kohlmeyer 1979; Kohlmeyer and Volkmann-Kohlmeyer 2003; Li and Wang 2009; Vala et al. 2016). In order to classify these fungi more generally, the term "marine-derived fungi" is widely used (Bonugli-Santos et al. 2015; Vala et al. 2016; Christophersen et al. 1998; Osterhage 2001). Strains of marine mycobiota have been found to inhabit almost all possible marine habitats ranging from inorganic matter, detritus, water, sediments, marine plants, and marine vertebrates to invertebrates and marine extreme environment. Marine-derived fungi have been observed to possess diverse potentials (Vala et al. 2004, 2016; Raghukumar et al. 1994; Vala 2010, 2018; Vala and Sutariya 2012; Vala and Dave 2015). However, comparatively less information is available on harnessing them for white biotechnology. Many pharmaceutical companies are focusing on extremophilic bacteria and involved in bioprospecting marine extreme environments; however, fungi from such environment despite their potentials have not been explored much for commercial applications (Raghukumar 2008). This chapter focuses on a few of the white biotechnologically relevant potentials of marine-derived fungi including some enzymes, biopesticides, and nutraceuticals (Fig. 15.1).

15.2 Enzymes from Marine-Derived Fungi

Marine-derived fungi are promising resources for biotechnologically important enzymes. In this section, a few of them, viz., xylanases, cellulases, L-asparaginases (LA), and lignin-modifying enzymes (LMEs) produced by marine-derived fungi, have been discussed.

15.2.1 Xylanases

One of the main constituent of hemicellulose, xylan comprises a chain of β-1,4-linked xylopyranose residues, and in nature, it is the second most abundant polysaccharide (Polizeli et al. 2005). Complete hydrolysis of xylan is brought about by an enzyme complex including endo-β-1, 4-xylanases, exoxylanases, and β-xylosidases,

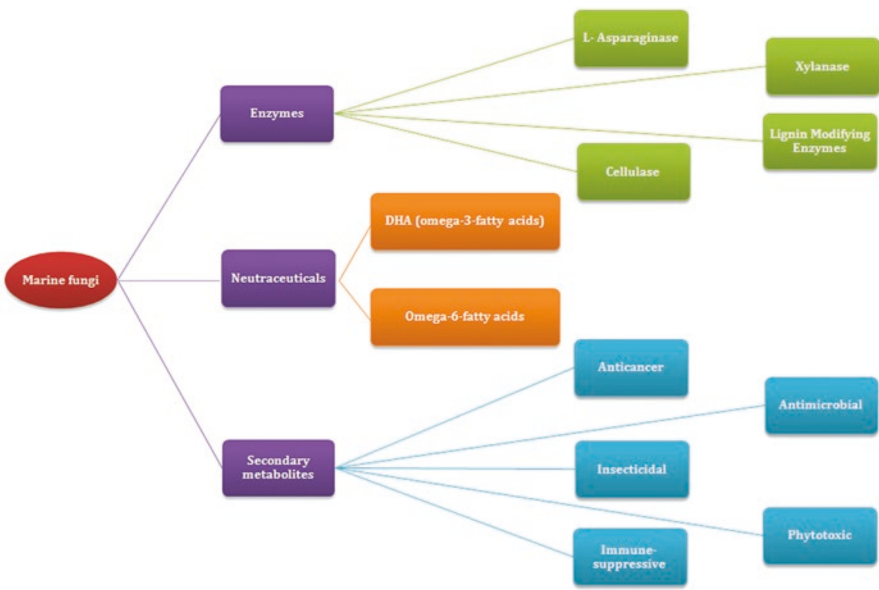

Fig. 15.1 Some ecologically and economically important compounds produced by marine fungi

liberating xylose monomers or oligomers. Bacteria, filamentous fungi and yeasts are among the main producers of these enzymes, especially the filamentous fungi are considered as better source for these enzymes (Kulkarni et al. 1999; Beg et al. 2001; Korkmaz et al. 2017).

Xylanases are a set of enzymes involved in xylan biodegradation, having the ability to hydrolyze the main chain of xylan to oligosaccharides and successively degrading it to xylose (Chávez et al. 2006; Santos et al. 2016).These enzymes are biotechnologically very promising. In various sectors including paper and pulp industries, as well as feed and food industries, xylanases play a big role in developing environmentally friendly technologies. Besides, they are also useful in producing liquid fuel and chemicals from lignocellulose (Juturu and Wu 2012; Santos et al. 2016). Currently, industrial production of xylanases is mostly carried out using members of genera *Aspergillus*, *Penicillium*, and *Trichoderma*. It has been suggested that fungi from marine environment do produce xylanases; however, they have not been studied in much as compared to their terrestrial counterparts (Yadav et al. 2018; Bonugli-Santos et al. 2015; Korkmaz et al. 2017; Santos et al. 2016).

Raghukumar et al. (2004) examined obligate and facultative marine fungi for xylanase production and concluded that facultative marine fungi (*Aspergillus niger* and *A. ustus*) were the best xylanase-producing isolates. The authors claim the study to be the first report revealing possible application of thermostable, cellulase-free alkaline xylanase activity from crude marine-derived fungal filtrate in bio bleaching of paper pulp. Marine-derived fungi isolated from marine macroorganisms, the sponges, the ascidians, and the algae from São Paulo State, Brazil, were screened for xylanase production potential (Santos et al. 2016). Out of 493 test fungi, 112

could degrade under experimental conditions. Among all isolates, largest numbers of xylanase-producing fungi were from marine sponges. *Aspergillus* cf. *tubingensis* LAMAI 31 was found to be the best xylanase producer (49.41 U/mL); upon optimization of cultural conditions, 12.7 times increase in xylanase production could be achieved. The enzyme was stable in the temperature range of 40–50 °C and in pH range from 3.6 to 7.0 (optimum temperature and pH 55 °C and 5.0, respectively). The authors suggested *A*. cf. *tubingensis L*AMAI 31 to be a new genetic resource for xylanase production.

Aspergillus sp. NRCF5exhibiting high xylanolytic activity was isolated from the inner tissue of Egyptian soft coral *Rhytisma* sp. (El-Bondkly 2012). While using the principles of genome shuffling in breeding of xylanase-producing fungi, this isolate was used as a starting strain, and genetic variability was induced using mutagens in different combinations and doses. A high xylanase-producing recombinant R4/31 with high stability was obtained that could produce 427.5 U/ml xylanase. Mostafa et al. (2014) examined presence of extracellular xylanase activity in extracts of 18 marine-derived fungi grown on wheat bran as solid-state fermentation medium for 10 days. Out of 18, only three isolates, viz. *Aspergillus flavus*, *Cladosporium sphaerospermum*, and *Epicoccum purpurascens*, exhibited extracellular xylanase activity. *A. flavus* was observed to be the most efficient among the three, as evidenced by zone of clearance on xylan agar plate. Further, the authors observed improved xylanase production by cocultivation of the three fungal isolates on wheat bran and sawdust at a ratio of 1.5:1.5 w:w. Harnessing the low-cost substrates, i.e. agro-wastes, would not only reduce the production cost but also reduce the environmental pollution; hence, the process would be industrially as well as ecologically important suggesting the marine-derived fungi to be promising candidates for white biotechnology.

Sixteen endophytic marine-derived fungi (eight from seagrasses and eight from seaweeds) were examined for their potential to produce xylan-degrading enzymes (Thirunavukkarasu et al. 2015). Agar plate assay revealed that 50% of the total isolates exhibited xylanase activity. Further investigations based on spectrophotometric assays revealed that *Trichoderma harzianum*, an endophyte from brown alga *Sargassum wightii*, exhibited maximum secretion of xylanase and xylosidase. Remarkable NaCl tolerance of *T. harzianum* and increase in extracellular xylanase and xylosidase activity in presence of 0.26 M NaCl in the medium highlighted importance of marine-derived fungi as a reservoir of enzymes, especially novel cell wall-degrading enzymes, relevant to biofuel industry.

Korkmaz et al. (2017) examined 88 marine-derived filamentous fungal strains (isolated from sponges and sediment samples from the coastal sides of Aegean and Mediterranean Seas of Anatolian Peninsula) for xylanase production ability. Extracellular xylanolytic activities were exhibited by 92% of the test isolates. From these, *Trichoderma pleuroticola* 08ÇK001 was selected for further studies, and xylanase activity of this isolate was further characterized. Resistance to various metal ions exhibited by *T. pleuroticola* 08ÇK001 xylanase could be considered as an added advantage for its industrial application. Based on the findings of *T. pleuroticola* 08ÇK001 xylanase activity, it was suggested that the marine-derived fungi, especially *T. pleuroticola* 08ÇK001, could be possibly employed in certain indus-

trial processes including juice and wine industries, animal feed, and paper pulping. *T. pleuroticola* 08ÇK001 xylanase could be a potential candidate for animal feed or juice and wine industries that require high stability and optimum activity at an acidic pH. Especially, resistance of the enzyme against presence of organic solvents enriches its fitness for paper pulping applications.

Torres and dela Cruz (2013) examined 44 mangrove fungi for their xylanolytic activity. Among the test isolates, 93% showed xylanolytic activity in solid medium. Further studies revealed that crude xylanases had optimum activity at temperature 50 °C and pH 7. pH was a more influential parameter than temperature. *Fusarium* sp. KAWIT-A and *Aureobasidium* sp. 2LIPA-M produced thermophilic xylanases. *Phomopsis* sp. MACA-J exhibited production of alkaliphilic xylanase. As revealed by liberation of high quantities of reducing sugars, mangrove fungal crude xylanases were observed to have potential applications in the local paper and pulp industry.

15.2.2 Cellulases

Cellulose, a simple polymer of glucose residues with β-1, 4-glycosidic linkages, is the most abundant carbohydrate produced by plants. Despite its structural simplicity, cellulose forms insoluble, crystalline microfibrils that are highly recalcitrant (Béguin and Aubert 1994; Shewale 1982). Cellulolysis can be brought about by cellulase enzyme complex system consisting of extracellular enzymes, viz., 1, 4-β-endoglucanase (catalyzes random cleavage of β-1, 4-glycosidic bonds along a cellulose chain), 1, 4-β-exoglucanase (cleavage of the nonreducing end of a cellulose chain and splitting of the elementary fibrils from crystalline cellulose), and β-glucosidase (β-D-glucoside glucohydrolase or cellobiase) (hydrolysis of cellobiose and water-soluble cellodextrin to glucose) (Shewale 1982; Woodward and Wiseman 1982). Hence, synergistic activity of these three enzymes leads to complete hydrolysis of cellulose (Ryu and Mandels 1980; Samdhu and Bawa 1992; Wood 1989; Gupta et al. 2012).

Cellulases have a big role to play in a range of industrial processes. Besides being important to manufacturing of paper and textiles and preparation of laundry detergents, recently, cellulases have gained attention due to their significance in development of biofuel production processes using renewable resources like cellulosic and lignocellulosic materials (Park et al. 2006). Comparatively high cost of cellulases is a major barrier in bulk fermentation processes for cellulosic biofuel (Zhang et al. 2006). Compared to chemical reactions, biocatalytic reactions for biofuel production lead to higher yield in environmentally friendly manner (Lee et al. 2013). Cellulases are produced by bacteria as well as fungi. Investigations on degradative enzyme production by fungi associated with detritus of the leaves of mangrove *Rhizophora apiculata* were carried out, and it was observed that all isolated fungi produced cellulases, while some of them could produce amylase, xylanase, pectin lyase, and protease also (Raghukumar et al. 1994).

Vala et al. (2000) studied four facultative marine fungi, viz., *Aspergillus nidulans*, *A. versicolor*, *Paecilomyces variotii*, and *Penicillium citrinum*, for cellulase production. Effect of media on cellulase production was also examined. All the test fungi exhibited production of exoglucanase, endoglucanase, and β-glucosidase as well as complete cellulase activity. Highest complete cellulase activity was exhibited by *A. versicolor*. Mandel and Sternberg's medium supported maximum enzyme production by all test fungi. Ravindran et al. (2010) screened marine-derived *Chaetomium* sp. for alkaline cellulase production and carried out production of these enzymes harnessing various wastes as substrates in the submerged and solid-state fermentation processes. Maximum enzyme secretion could be obtained with cottonseed as substrate under solid-state fermentation conditions at high alkaline pH. The produced enzymes exhibited high stability and activity. Characterization of enzymes further emphasized on their white biotechnological applications.

Chaetomium globosum, an alkaline-tolerant marine-derived fungus, was examined for enhanced production of cellulases and free phenolics under highly alkaline conditions using substrates cottonseed and sugarcane bagasse under solid-state fermentation processes (Ravindran et al. 2011). The authors observed an increase in production of cellulases with increase in pH in case of both the agro-wastes. High phenolic release was also observed at higher pH. The total released phenolic contents and total antioxidant property exhibited a linear correlation. Based on the study, it was suggested that using agro-wastes, cost-effective production of nutraceutical ingredients is possible.

Ghazala et al. (2016) screened five marine-derived fungi (*Aspergillus niger*, *A. flavus*, *Penicillium oryzae*, *P. chrysogenum*, and *Rhizopus oryzae*) for cellulase production in order to break down the algal cell wall. Highest cellulase activity was observed in the case of *A. niger*; when applied for biodiesel production, performance of crude extract of marine-derived *A. niger* was observed to be better than that of commercially available cellulase. Santos et al. (2017) examined four marine-derived fungal strains, viz., *Penicillium citrinum* CBMAI1186, *Aspergillus sydowii* CBMAI 934, *Aspergillus sydowii* CBMAI935, and *Mucor racemosus* CBMAI 847, for production of cellulases under solid-state fermentation. Cultural conditions as well as efficiency of the cellulases in hydrolysing cellulose from sugarcane bagasse were also studied. Rind and pith fractions of sugarcane bagasse with and without alkali pretreatment were used for cellulose hydrolysis. The untreated bagasse was observed to be invariably resistant to saccharification. In the rind and pith, respectively, 56% and 81% saccharification could be achieved using cellulases from *A. sydowii* CBMAI 934.

Ramarajan and Manohar (2017) developed consortia of potential cellulolytic and ligninolytic marine-derived fungal isolates in order to determine their capability for biodegradation of the lignocellulosic substrates like rice straw and sugarcane bagasse. Based on various analyses, the authors proved that the marine-derived fungal consortia have application potential for effective exploitation of agricultural wastes. Batista-García et al. (2017) examined lignocellulolytic activities of 14 marine-derived fungi isolated from the deep-sea sponge *Stelletta normani*. High CMCase and xylanase activities were exhibited by three isolates *Cadophora* sp.,

Emericellopsis sp., and *Pseudogymnoascus* sp. When grown on corn stover and wheat straw in solid-state fermentation, these three fungi could produce CMCases, xylanases, and peroxidase/phenol oxidases. Fungal communities associated with deep-sea sponges were envisaged as potential source of novel biocatalysts; especially lignocellulose degradation potential was suggested important looking to the need for improved biomass conversion strategies.

Baker et al. (2010) also reported marine-derived fungi associated with marine sponges as potential source for novel cellulase activities. Just as filamentous marine-derived fungi, yeasts from marine environment have also been observed to exhibit biotechnologically relevant potentials. Duarte et al. (2013) reported Antarctic marine yeasts isolated from marine samples like different marine invertebrates and sediments to produce cellulases, lipases, and proteases at 15 °C on solid media.

15.2.3 L-Asparaginase

L-asparaginase (LA) is a commercially important enzyme. It is used in treatment of certain cancers, especially acute lymphoblastic leukemia (ALL) in children. It is also used in food industry. L-asparagine is irreversibly hydrolyzed to L-aspartic acid and ammonia by LA. While the normal cells remain unaffected in the presence of LA, tumor cells being auxotrophic to L-asparagine undergo nutritional stress ultimately leading to apoptosis (Hendriksen et al. 2009; Anese et al. 2011; Husain et al. 2016). LA used as an anticancer agent is commercially available from bacterial sources. However, it's suffering from certain limitations, and searching for newer sources of LA could be a solution to this problem. LA has also been a promising alternative to alleviate acrylamide (a potent carcinogen) formation during bakery goods production. LA of fungal origin is used in food industry (Hendriksen et al. 2009; Mahajan et al. 2012; Huang et al. 2013). Marine-derived fungi have been reported to produce LA (Vala and Dave 2015). However, their LA production potentials have not been explored much for their white biotechnological applications.

Farag et al. (2015) isolated 21 marine-derived fungal strains from Red Sea coasts of Egypt. Out of them five fungal strains belonging to genera *Aspergillus*, *Penicillium*, and *Fusarium* were screened for LA production. *Aspergillus terreus* was found to be the most efficient isolate showing maximum LA specific activity (4.81 U/mg protein). Immobilization on sponge enhanced the LA production efficiency of *A. terreus* by 1.33-fold.

Murali (2011) screened endophytic fungi isolated from algae from southern coast of Tamil Nadu for LA production. Sixty four out of 82 fungi were LA positive. *Fusarium* sp. and a sterile mycelial form showed maximum LA activity. Highest enzyme production was achieved on day 5 and optimum pH was found to be 6.2. Izadpanah et al. (2018) have reviewed LA from different marine microbial sources including marine-derived fungi and have appraised marine LA as potential candidates for medical and industrial applications. The authors have mentioned that properties of LA from marine-derived fungi have comparatively been less reported.

However, the reports available suggest the properties of LA to be important for industrial applications. Recently, LA from marine-derived fungi from Bhavnagar Coast, Gulf of Khambhat, west coast of India, has been studied in detail at Maharaja Krishnakumarsinhji Bhavnagar University. It has been reported that 70% of marine-derived fungal isolates exhibited LA activity (Vala and Dave 2015). Vala and Dave (2015) examined 20 marine-derived fungi from Gulf of Khambhat, west coast of India, for LA production. Fourteen test fungi exhibited LA production. *Aspergillus niger* emerged as the strongest producer followed by *A. terreus*.

Vala et al. (2018a) carried out sequential optimization of medium components to enhance LA production potential of a euryhaline marine-derived fungus *Aspergillus niger* strain AKVMKBU. With the help of a Plackett-Burman design followed by response surface methodology using central composite design, 73.52% rise in LA activity could be achieved. To further improve LA production, optimization of pH, incubation time, and inoculum size was carried out with the help of process-centric (response surface methodology [RSM]) and data-centric (artificial neural network [ANN]) approaches (Vala et al. 2018b). For LA production by a marine-derived fungus, detailed analyses for RSM and ANN models have been reported for the first time. Upon purification and characterization of LA from marine-derived AKV-MKBU, it was revealed that the enzyme having molecular weight ~90KDa retained activity over pH range 4–10 and Tween 80 and Triton X-100 enhanced the LA activity, while heavy metals reduced the enzyme activity (Vala et al. 2018c). Noteworthy antiproliferative activity of LA against various cancer cell lines was observed. Using groundnut oil cake as low-cost substrate, bench-scale production of LA was also carried out. Hence, marine-derived fungi can be viewed as potential candidates for white biotechnological production of LA.

15.2.4 Lignin-Modifying Enzymes (LMEs)

Synthetic textile dyes contain compounds such as azo, anthraquinone, triphenylmethane, and heterocyclic polymeric structure among which azo dyes are the largest and most versatile class of dyes (Diwaniyan et al. 2010). When released in aquatic environment in the form of industrial effluents, these dyes can obstruct photosynthesis and the diffusion of gasses making them a great concern for aquatic and human health (Baughman and Weber 1994; Ciullini et al. 2008).

With regard to their high recalcitrant nature, considerable efforts have been made toward making a cost-effective and successful treatment for the removal of wastewater dyes. Microbial bioremediation has been considered as an appealing biotechnological alternative for treating hazardous compounds like dyes (Bonugli-Santos et al. 2015). Since a large number of effluents generated through textile processes have saline and alkaline conditions, marine fungi demonstrate an important biological advantage for decolorization/degradation of these effluents because of their adaptability to high salt and pH (Ciullini et al. 2008). Among the extracellular enzymatic machineries produced by filamentous fungi, the ligninolytic enzyme sys-

tem is of great significance in environmental remediation of components like dyes (Arun et al. 2008). Many researchers have reported significant decolorization of textile effluents and synthetic dyes, e.g. Congo Red, Brilliant Green, and Remazol Brilliant Blue R (RBBR), by marine-derived fungi. Chen et al. (2014) have studied a whole-cell immobilization system using marine-derived fungi *Pestalotiopsis* sp. J63 and *Penicillium janthinellum* P1 and have demonstrated good ability of these fungi to decolorize Azure B dye.

Ligninolytic enzymes are collectively termed as lignin-modifying enzymes (LMEs) which comprise a variety of classes of extracellular enzymes, namely, lignin peroxidases, manganese peroxidases, laccases, and versatile peroxidases. These enzymes can degrade or modify not only lignin but also several recalcitrant, aromatic pollutants such as those occurring in oil waste, textile dye effluents, and organochlorides from agrochemical waste which are the sources of critical environmental pollution (Kiiskinen et al. 2004). Marine mangrove fungi have proved to be potential producers of ligninolytic enzymes (Raghukumar et al. 1994; Pointing and Hyde 2001). Majority of the ligninolytic marine fungi are soft-rot fungi with relatively few white-rot fungi (Pointing and Hyde 2001). Raghukumar et al. (1994) have confirmed the presence of laccase, cellulase, etc. from several obligate and marine-derived mangrove-associated fungi. Laccase was found to be widely distributed in marine fungi found on decaying lignocellulosic material in marine environment, while LiP and MnP were observed to be relatively less common in these fungi. D'Souza-Ticlo et al. (2006) have isolated marine-derived white-rot fungi from decaying mangrove wood which was found to be producing enhanced levels of laccase in the presence of several phenolic and lignin derivatives. However, Verma et al. (2010) have reported production of laccase in marine-derived fungi belonging to *Ascomycetes* and *Basidiomycetes* which demonstrated decolorization and detoxification of textile industrial effluents.

Apart from decolorization and detoxification of textile dye effluents, LMEs can also be employed for degradation of polycyclic aromatic hydrocarbons (PAHs), one of the major components of crude and petroleum wastes and one of the most concerned pollutants of marine ecosystems as well. LiPs are heme-proteins of the secondary metabolism of fungi such as white-rot fungi. Due to a higher redox potential than other peroxidases and oxidases, LiP can oxidize a wide range of environmentally persistent pollutants having high ionization potential values such as PAHs (Ward et al. 2003). MnPs are also nonspecific heme-proteins production of which is induced by Mn^{2+}. It is produced by very few species belonging to ascomycetous and basidiomycetous fungi (Hofrichter et al. 1998; Lopez et al. 2007). Unlike other peroxidases, MnPs oxidize Mn^{2+}, a preferred electron donor into Mn^{3+} which subsequently mediates the oxidation of a variety of amorphous molecules, i.e. lignin or other phenolic as well as non-phenolic compounds, including PAHs (Hofrichter 2002). Laccases are phenoloxidases constituting a family of multi-copper oxidases that catalyze the oxidation of a wide range of organic compounds. Having broad substrate preference and nonspecific catabolism to reduce an array of compounds, these enzymes can be exploited for biodegradation of organic pollutants including PAHs. There are many reports on degradation of PAHs by laccase.

Pozdnyakova et al. (2006) have studied degradation of anthracene, phenanthrene, fluorene, pyrene, and fluoranthene by laccase of white-rot fungus *Pleurotus ostreatus* D1 under the presence of synthetic mediator. VPs are able to oxidize Mn^{2+} directly and can oxidize aromatic substrates similar to that of MnP and LiP. It is postulated that catalytic nature of few peroxidase is due to a hybrid molecular architecture combining different substrate-binding and oxidation sites (Camarero et al. 2000; Wu et al. 2010). Since there are very few rather negligible reports on degradation of PAHs by LMEs from marine fungi, there are good opportunities in this specific area of research.

As LMEs are able to degrade a wide variety of substrates via free radical-mediated oxidizing reactions, these enzymes are also considered of great importance in the biofuel field, due to the possible resistance and activity in the presence of solvents and different pH conditions (Bonugli-Santos et al. 2015). Intriago (2012) has mentioned the prospect of utilizing marine microorganisms in cellulosic ethanol production. However, there are negligible reports related to the use of marine-derived fungi or their enzymes for ethanol production (second generation) in the available literature. Thus, marine fungi should be considered as the target in studies related to industrial and environmental applications (Chung et al. 2000), including the biological treatment of lignocellulosic substrate for biofuel production and PAHs bioremediation. Table 15.1 depicts some commercially important enzymes produced by marine fungi.

Table 15.1 Enzymes produced by marine-derived fungi and their source of isolation

Enzyme	Fungi	Source	Reference
Protease	*Aureobasidium pullulans*	Saltern sediment	Chi et al. (2007)
Amylase	*Mucor* sp.	Sponge *Spirastrella* sp.	Mohapatra et al. (1998)
Laccase	*Cerrena unicolor*	Decaying mangrove wood	D'Souza-Ticlo et al. (2009)
	Mucor racemosus CBMAI 847	Cnidarian *Mussismilia hispida*	Bonugli-Santos et al. (2010a)
	Marasmiellus sp. CBMAI 1062	Sponge *Amphimedon viridis*	Bonugli-Santos et al. (2010b)
	Peniophora sp. CBMAI1063	Sponge *Amphimedon viridis*	Bonugli-Santos et al. (2010b)
Lipase	*Geotrichum marinum*	Marine soil	Huang et al. (2004)
Lignin peroxidase	*Mucor racemosus* CBMAI847	Cnidarian *Mussismilia hispida*	Bonugli-Santos et al. (2010a)
	Tinctoporellus sp. CBMAI 1061	Sponge *Dragmacidon reticulata*	Bonugli-Santos et al. (2010b)
Manganese peroxidase	*Mucor racemosus* CBMAI847	Cnidarian *Mussismilia hispida*	Bonugli-Santos et al. (2010a)
Xylanase	*Aspergillus niger*	Mangrove leaf detritus	Raghukumar et al. (2004)
L-asparaginase	*Aspergillus niger*	*Avicennia marina*	Vala et al. (2018a)
Cellulase	*Aspergillus versicolor*	Water	Vala et al. (2000)

Adopted and modified from Bonugli-Santos et al. (2015)

15.3 Bioactive Compounds from Marine-Derived Fungi

Apart from numerous enzymes, fungi from marine environment have proved to be a rich source of novel biological natural products. Because of their unique habitat with reference to temperature, nutrients, competition, and salinity, marine fungi have developed specific pathways for secondary metabolism compared to terrestrial fungi (Liberra and Lindequist 1995). These secondary metabolites often show pharmaceutically significant bioactivities and may prove to be candidates for the development of new drugs. From 2006 to 2010, a total of 690 natural products from fungi in marine habitats had been reported (Katia et al. 2012), and increase in the number still continues. Some of the important bioactive secondary metabolites with their producer fungi and applications are summarized in Table 15.2. Members of the genera *Penicillium* and *Aspergillus* were major candidates in this field as they produced most of the novel compounds (Blunt et al. 2015). However, the diversity of marine fungi has by far not been satisfactorily represented in the studies related to marine natural products. Bringmann et al. (2005) have isolated sponge-derived *Penicillium chrysogenum* and have reported production of sorbicillactones A and B, which were considered as specifically active against human leukemia cell lines. Along with these two, a number of other derivatives of sorbicillin including 6-hydr oxyoxosorbicillinol,oxosorbicillinol, sorbifuranol, sorbivineton, and bisvertilonon were also observed. Marine representatives of the genus *Trichoderma* produce a variety of bioactive metabolites, such as the antimycobacterial aminolipopeptide trichoderins (Pruksakorn et al. 2010), the antifungal trichodermaketone A (Song et al. 2010), the cytotoxic dipeptide trichodermamide B (Garo et al. 2003), and antibacterial tetrahydroanthraquinone and xanthone derivatives (Khamthong et al. 2012; Ruiz et al. 2013).

Marine isolates of the genus *Stachybotrys* have been obtained from various marine environments such as the rhizosphere of mangroves, mud of the intertidal zone, intertidal pools, brackish waters, marine sediments and sponges, marine algae, and sea fans (Wu et al. 2014; Gupta et al. 2007). Spirocyclic drimanes represent a major class of secondary metabolites produced by *Stachybotrys* species (Jarvis 2003). Spirocyclic drimanes are associated with a number of different biological activities, such as immunosuppressive activity (Kaise et al. 1979), endothelin receptor antagonistic activity (Ogawa et al. 1995), and inhibition of tyrosine kinase (Vázqueza et al. 2004).

Talaromyces funiculosum of marine origin is also known as producer of a number of bioactive compounds (Wu et al. 2015), such as lovastatin inhibiting the 3-hydroxy-3-methylglutaryl-coenzyme A reductase, an important enzyme in the biosynthesis of cholesterol (Seydametova et al. 2012). Lovastatin is an approved drug. In addition to this, secalonic acid D with cytotoxic activity; 11-desacetoxy-wortmannin, a fungicidal and anti-inflammatory metabolite; and helenin which is active against the swine influenza virus were also identified from *Talaromyces funiculosum* (Imhoff 2016).

Table 15.2 Secondary metabolites produced by marine fungi and their application in biomedical field

Fungus	Secondary metabolite	Activity/application	Reference
Penicillium citrinum	Alkaloid	Anticancer compound	Tsuda et al. (2004)
Fusarium sp.	Cyclic tetrapeptide	Anticancer	Ebel (2010)
Apiospora montagnei	Diterpene	Activity against human cancer cell lines	Klemke et al. (2004)
Scytalidium sp.	Hexapeptide	Inhibitor of herpes simplex virus	Rowley et al. (2003)
Penicillium chrysogenum	Sorbicillactones A and B	Active against human leukemia cell lines	Bringmann et al. (2005)
Trichoderma sp.	Trichoderins	Antimycobacterial	Pruksakorn et al. (2010)
Trichoderma sp.	Trichodermaketone A	Antifungal	Song et al. (2010)
Trichoderma sp.	Trichodermamide B	Cytotoxic dipeptide	Garo et al. (2003)
Trichoderma sp.	Tetrahydroanthraquinone	Antibacterial	Khamthong et al. (2012)
Trichoderma sp.	Xanthone derivatives	Antibacterial	Ruiz et al. (2013)
Stachybotrys sp.	Spirocyclic drimanes	Immunosuppressive activity	Kaise et al. (1979)
		Endothelin receptor antagonistic activity	Ogawa et al. (1995)
		Inhibition of tyrosine kinase	Vázqueza et al. (2004)
Talaromyces funiculosum	Lovastatin	Inhibition of 3-hydroxy-3-methylglutaryl-coenzyme A reductase	Seydametova et al. (2012)
Bartalinia robillardoides	Taxol	Anticancer	Gangadevi and Muthumary (2008)
Cladosporium sp.	Cladosporides	Antifungal	Hosoe et al. (2000)
	Cotylenins	Plant growth factors	Sassa (1971)
	Cladosporin	Antifungal, antibacterial, insecticidal, phytotoxic, and immunosuppressive properties	Imhoff (2016)

Adopted and modified from Raghukumar (2008)

Species from the genus *Bartalinia* is rare among marine fungi (Wiese et al. 2011). *B. robillardoides* is a known producer of an anticancer drug in clinical application called taxol (Gangadevi and Muthumary 2008). *Cladosporium* is one of the largest and most heterogeneous fungal genera (Bensch et al. 2012) occurring ubiquitously in marine habitats. Various species of *Cladosporium* have been shown to produce a variety of natural products, for instance, melanin pigments, antifungal cladosporides (Hosoe et al. 2000), cotylenins, the plant growth factors

(Sassa 1971), calphostins, inhibitors of the protein kinase C (Kobayashi et al. 1989), and cladosporin which exhibits a variety of activities like antifungal, antibacterial, insecticidal, phytotoxic, and immunosuppressive properties (Imhoff 2016).

Marine-derived fungi have also gained attention as promising candidates for pest control, and they could be utilized in integrated pest management strategies (Thatoi et al. 2013). Xiao et al. (2005) reported nematicidal activity of secondary metabolites produced by marine-derived fungi. Cheng et al. (2008) reported a mangrove fungus having ability to produce insecticidal metabolites against several important pests. Swe et al. (2009) explored mangrove habitat of Hong Kong and reported 31 fungal isolates belonging to genera *Arthrobotrys*, *Monacrosporium*, and *Dactylella* to possess nematode-trapping ability. Recently, Pacheco et al. (2017) evaluated marine-derived fungi *Aspergillus versicolor*, *A. sydowii* (isolates 1 and 2), *Penicillium dipodomyicola*, and *Trichoderma harzianum* for the control of the aphid *Brevicoryne brassicae*. Among these, *A. versicolor* was observed to be the most effective as revealed by 85.9% mortality in *B. brassicae* at 24 h. Entomopathogenic efficiency of *A. versicolor* was comparable to commercially available bioinsecticides formulated using *Beauveria bassiana* (Bovemax®) and *Metarhizium anisopliae* (Methamax®). The authors suggested the study to be the first to demonstrate role of marine-derived *A. versicolor* as a potential biocontrol candidate for agricultural pest.

15.4 Nutraceuticals from Marine-Derived Fungi

Microbial oils rich in omega-3-polyunsaturated fatty acids (PUFAs) are one of the major commercial biotechnology products nowadays. Many studies during the 1970s have indicated the docosahexaenoic acid (DHA), an omega-3 PUFA, is an essential fatty acid providing cardiovascular health (Raghukumar 2017). Marine fungi have also been known to be a potential source of carotenoids; omega-3 fatty acids, including DHA and EPA (Pino et al. 2015); and omega-6 fatty acids like arachidonic acid (Iida et al. 1996). Marine fungi, namely, *Trichoderma* sp. and *Rhodotorula mucilaginosa* AMCQ8A, are known to produce DHA-rich oil. A yeast species isolated from seawater, *Rhodotorula mucilaginosa* AMCQ8A, is capable of producing high biomass with high lipid yield (Kot et al. 2016). Marine fungoid protist *Schizochytrium* belonging to *Labyrinthulomycetes* is a rich source of DHA-rich oil. Human nutraceuticals comprising of *Schizochytrium* oil were marketed by Omega Tech and Martek Biosciences in the USA. *Thraustochytrids*, a fungus-like *Stramenopiles*, is now becoming a significant source of PUFAs for biotechnological industries (Leyland et al. 2017). Fungi from marine environment have been proven to be a great reservoir of battery of novel ecologically and economically important compounds; however, their potentials are quite untapped if considered for their

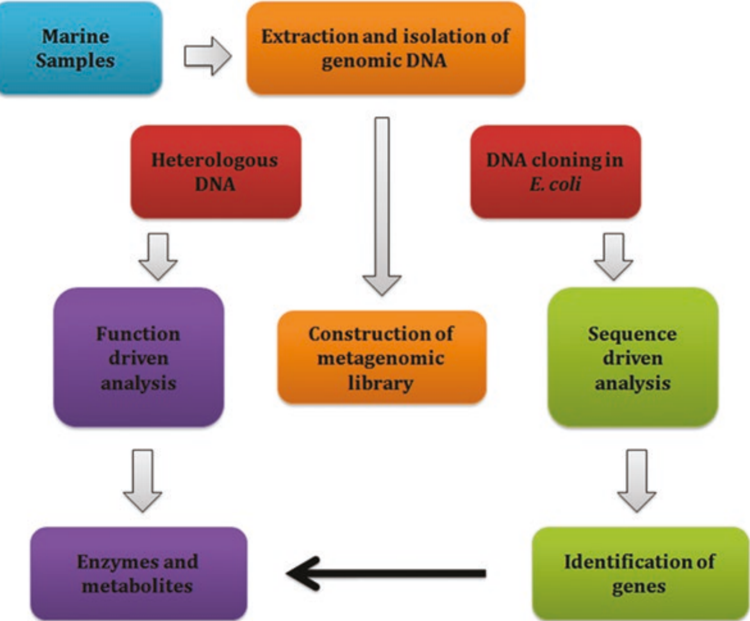

Fig. 15.2 Metagenomic approach for exploitation of fungi from marine environment

commercial utilization. Hence, these group of fungi demand attention. Figure 15.2 illustrates the metagenomic approaches for exploitation of marine fungal bioactive compounds.

15.5 Conclusion and Future Prospects

Today our knowledge about the fungal white biotechnology has evolved greatly where many of the bioactive compounds including enzymes from marine fungi have proved to be more efficient, more sustainable, and cost-effective than chemically synthesized products. Still there is scope for future research in this field. The following points can be of prime importance for improvement in the future studies related to the bioactive compounds from marine fungi. Deep-sea fungal diversity may prove to be the source of more effective and novel bioactive compounds and, thus, needs to be explored further. Evolution of biosynthetic pathways of the fungi from marine environment and their regulation should be studied in order to understand the formation of various compounds. Modern molecular techniques like molecular crystallography, enzyme modulation, and molecular characterization in combination with the classical enzymology methods could be helpful to characterize novel enzymes and to study their functions. Use of metabolomic and genomic

approaches can be employed to assess the functional and phylogenetic diversity of the marine fungi at genus and species level. A whole new treasure of natural bioactive compounds can be obtained if these prospects are applied systematically in the search of novel bioactive compounds from marine-derived fungi.

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Chapter 16
Discovery of New Extremophilic Enzymes from Diverse Fungal Communities

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Abstract Extremophiles are microorganisms that are found in environments of extreme temperature (−2–15, 60–110 °C), ionic strength (2–5 M NaCl), or pH (<4, >9). Extremophiles are a source of enzymes (extremozymes) with extreme stability, and the application of these enzymes as biocatalysts has attracted attention because they are stable and active under conditions at which the normal enzymes do not work. These microorganisms are capable of surviving under extreme conditions in non-conventional environments, and their enzymes are adapted to these conditions. The properties of their enzymes have been optimized for these conditions. Extremophiles, particularly those from the Archaea, have novel metabolic pathways; hence, they serve as a source of enzymes with novel activities and applications. Among eukaryotes, fungi are the most versatile and ecologically successful phylogenetic lineage. With the exception of hyperthermophily, they adapt well to extreme environments. Fungi are found to live in acidic and metal-enriched waters from mining regions, alkaline conditions, hot and cold deserts, and the deep ocean and in hypersaline regions such as the Dead Sea. Extremophilic enzymes from fungi have been sought for because of the increasing industrial demands for biocatalysts that can cope with industrial process needs. Extremozymes have a great economic potential in many industrial processes, including agricultural, chemical, and pharmaceutical applications.

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16.1 Introduction

The extremophiles thrive in habitats which are intolerable for other terrestrial life forms. They grow well in extreme hot niches, ice, and salt solutions, as well as acid and alkaline conditions. Some extremophiles grow in organic solvents, heavy metals, or in several other habitats that were previously considered inhospitable. Extremophiles have been found at depths of 6.7 km inside the Earth's crust, more than 10 km deep inside the ocean, at pressures of up to 110 MPa, from extreme acid (pH 0) to extreme basic conditions (pH 12.8), and from hydrothermal vents at 122 °C to frozen sea water, at −20 °C. They are classified according to the conditions in which they grow as (a) thermophiles and hyperthermophiles, (b) psychrophiles, (c) acidophiles and alkaliphiles, (d) barophiles, and (e) halophiles. In addition, these organisms are normally polyextremophiles (Yadav et al. 2015). They live in habitats with various physicochemical parameters at extreme values. For example, the deep sea has temperature, salinity, and pressure extremes. Thus this group includes prokaryotes, eukaryotes, & Archaea.

The extremophiles can be divided into two categories dependent on tolerance to the extremities. Extremophilic organisms necessitate, the extreme conditions for growth and extremotolerant organisms can grow optimally at normal values of physicochemical parameters but can tolerate the extremities of one or more of the same. Capece et al. (2013) tabulated over 200 extremophile species in an effort to provide a comprehensive look at the extremes of temperature and pH. The most versatile among the eukaryotes are the fungi. Except for extremities of high temperature, these fungi are well adapted to all extremities of the environment. This adaptation is known to be due to well adapted biomolecules and the metabolic pathways evolved in these species. These are of great biotechnological importance especially the enzymes, which are stable and active under extreme conditions. They are thus a good alternative for the labile mesophilic enzymes from the industrial point of view. Interestingly, some of these enzymes display polyextremophilicity (i.e., stability and activity in more than one extreme condition) that make their wide use in industrial biotechnology possible (Kvesitadze et al. 2012; Pabulo 2013; Shafer et al. 2000; Viikari et al. 2007; Yadav et al. 2016a, b, 2017a).

Some workers have reported the unique features of these enzymes such as being extremely thermostable and usually resistant against chemical denaturants such as detergents, chaotropic agents, organic solvents, and extremes of pH (Leuschner and Antranikian 1995; RuÈ digger et al. 1995; Friedrich and Antranikian 1996; Jùrgensen et al. 1997). Microorganisms such as thermophiles, halophiles, acidophiles, alkaliphiles, etc. often are capable to produce enzymes exceeding in stability of the currently used ones (Maheshwari et al. 2000; Pabulo 2010; Sahay et al. 2017; Saxena et al. 2016; Singh et al. 2016; Suman et al. 2015). The various enzymes secreted by extremophilic fungi along with the temperature maxima are compiled in Table 16.1. They can hence be used as models for designing and constructing proteins with new

Table 16.1 Extremophilic fungi and their enzymes with temperature optima for enzyme activity

Name of microorganism	Enzyme	Temperature	References
T. lanuginosus IlSc 91	Glucoamylase α-Amylase	50 °C	Mishra (1994)
Thermomyces lanuginosus	Amylase	50 °C	Kunamneni et al. (2005)
Malbranchea sulfurea	α-Glucosidases	60 °C	Gautam and Gupta (1992), Gupta and Gautam (1993)
Lipomyces starkeyi	α-Glucosidase	60 °C	Kelly et al. (1985)
			Costantinho et al. (1990)
Pyrococcus furiosus	α-Glucosidase	105–115 °C	Costantinho et al. (1990)
Rhizomucor pusillus	Glucoamylase	65 °C	Kanlayakrit et al. (1987)
Absidia corymbifera, *Gilmaniella humicola*, *Talaromyces helicus*, *Chaetomium elatum*, *Chaetomium* sp., *Humicola* sp., and *Rhizomucor pusillus*	Amylases	45 °C	Olagoke (2014)
T. aurantiacuss	Cellulases	46–51 °C	Romaneli et al. (1975)
S. thermophile		36–43 °C	
T. aurantiacus		48 °C,	
S. thermophile		40 °C	
C. thermophile		40 °C	
Chaetomium thermophile	Endoglucanase	28 °C	Nairn and Jarnil (2007)
T. reesei	Cellulases	45 °C	Wojtczak et al. (1987)
Thielavia terrestris, *Aspergillus terreus*, *Sporotrichum thermophile* QM-9382, *Thermoascus aurantiacus*	Cellulases	50–70 °C	Wojtczak et al. (1987), Romaneli et al. (1975)
T. aurantiacus	β-glucosidases	70 °C	Gomes et al. (2000)
Geotrichum sps and *Rhodotorula* sps	Cold-active lipase	0–30 °C	Divya and Naga Padma (2014)
Rhodococcus erythropolis N149	Lipase	15 °C	Maharana and Singh (2018)
Geotrichum and *Rhodotorula* sps	Lipase	25 °C	Divya and Padma (2015)
Myceliophthora sp.	Alkaline protease	40–45 °C	Zanphorlina et al. (2011)
Botrytis cinerea	Pectinase	40 °C	Pan et al. (2014)

properties that are of interest for industrial applications (Niehaus á et al. 1999). Present chapter deals with the extremophilic enzymes of extreme fungi discovered over the period of time and their significance. It highlights the various new findings that have contributed in the elaborate study of different enzymes produced by the fungal community as a whole.

16.2 Extremophilic Enzymes from Fungal Communities

16.2.1 Amylases

Thermophilic fungi are potential sources of enzymes with scientific and profitable interests. Enzymes of thermophilic fungi include cellulase, amylase, xylanase, polygalacturonase, glucoamylase, protease, lipase, α-amylase, cellobiose dehydrogenase, phytase, and glucosyl transferase. EMedia that can be used for the growth of thermophilic fungi are potato dextrose agar, starch-yeast extract agar, malt extract agar, and Sabouraud Dextrose Agar. Mechanisms of the thermostability and catalysis have been elucidated using the genes of thermophilic fungi encoding lipase; protease, xylanase, and cellulase have been obtained for elucidation of the mechanisms of their intrinsic (Maheshwari et al. 2000). Thermophilic fungi have a minimum growth temperature of or above 20 °C and a maximum growth temperature extending up to 60–62 °C (Maheshwari et al. 2000).

Thermophilic fungi are the only eukaryotic organisms that can grow at temperatures above 45 °C; they are valuable experimental systems for studying the mechanisms that allow growth at moderately high temperature yet limit their growth beyond 60 to 62 °C (Allen and Emerson 1949). Various genera of this group include the *Phycomycetes*, *Ascomycetes*, *Fungi Imperfecti*, and *Mycelia sterilia* (Mouchacca 1997). During the last four decades, many species of thermophilic fungi sporulating at 45 °C have been reported as cited in Abdullah and Zora (1993).

A strain of *T. lanuginosus* IISc 91 was isolated from a manure heap and was found to produce higher levels of extracellular amylolytic enzymes. This strain produced 4 units of glucoamylase and 40 units of α-amylase per ml of culture filtrate in the presence of 2% starch at 50 °C. The purified α-amylase was a homodimeric protein of 40 kDa having 5% (w/w) carbohydrate. The enzyme liberated oligosaccharides from starch with maltose being the principle product of hydrolysis. The Km for soluble starch was 2.5 mg ml^{-1}. A high Vmax of 8000 mg starch min^{-1} mg $protein^{-1}$ was found (Mishra 1994).

Later this same strain was reported to secrete one form each of α-amylase and glucoamylase during growth. Both enzymes were purified to homogeneity by ion-exchange and gel-filtration chromatography and obtained in mg quantities. α-Amylase, a dimeric protein of ~42 kDa, contained 5% (by mass) carbohydrate. It was maximally active at pH 5.6 and at 65 °C. The apparent K_m for soluble starch was 2.5 mg ml^{-1}. The enzyme produced exceptionally high levels of maltose from raw potato starch.

At 50 °C, this enzyme was stable for >7 h. At 65 °C, α-amylase was nearly eight times more stable in the presence of calcium. Addition of calcium increased the melting temperature of α-amylase from 66 to 73 °C. Upon incubation at 94 °C, α-amylase was progressively and irreversibly inactivated and converted into an inactive 72 kDa trimeric species (Mishra and Maheshwari 1996). The occurrence of thermophilic fungi in aquatic sediment of lakes and rivers as first reported by Tubaki et al. (1974) is indeed intriguing in view of the low temperature (6–7 °C) and low level of oxygen (average 10 ppm) conditions in these lakes.

Unal (2015) has described the isolation of thermophilic fungi from water and soil samples, producing amylase. The samples were obtained from the area where the thermal springs range from 55 to 90 °C around Afyon and Eskisehir in Turkey. Thermostable amylase activity of *Aspergillus niger*, *Aspergillus oryzae*, and *Aspergillus terreus* were evaluated. Haki and Gezmu (2012) reported a survey in six Ethiopian hyperthermal springs (Arbaminch, Awassa, Nazret, Shalla and Abijata, Wendo Genet, and Yirgalem) in order to assess the existence of thermostable α-amylase-producing thermophilic fungi and eubacteria. Further tests on the activity of amylases extracted from selected organisms at elevated temperatures were conducted to detect the best thermostable enzyme from these sources.

The production of extracellular amylase by the thermophilic fungus *Thermomyces lanuginosus* was studied in solid-state fermentation (SSF) (Kunamneni et al. 2005). Solid substrates such as wheat bran, molasses bran, rice bran, maize meal, millet cereal, wheat flakes, barley bran, crushed maize, corncobs, and crushed wheat were used as substrate for enzyme production. Growth on wheat bran yeilded the highest amylase activity. The maximum enzyme activity obtained was 534 U/g of wheat bran under optimum conditions of an incubation period of 120 h; an incubation temperature of 50 °C, with an initial moisture content of 90%; a pH of 6.0; an inoculum level of 10% (v/w); a salt solution concentration of 1.5:10 (v/w); and a ratio of substrate weight to flask volume of 1:100 with soluble starch (1% w/w) and peptone (1% w/w) as supplements. Gaur et al. (1993) investigated amylase production by *Humicola* and *Paecilomyces* species in different media; starch-yeast extract medium was the most suitable for both fungi. The amylase synthesis in both fungi was inducible by starch. Presence of antibiotics in the medium was also found to affect enzyme production. Streptomycin favored enzyme production by *Humicola* sp. in this medium at concentrations of up to 75 μ/ml, whereas with *Paecilomyces* sp. amylase production was adversely affected at above μm/mL streptomycin.

Humicola and *Paecilomyces* species showed temperature optima of 45 and 35 °C with 8 and 6 days' incubation, respectively, for amylase production which are not as significant; however, *Humicola* enzyme was thermally stable up to 50 °C.

Bo and Jørgen (1992) have described alpha-amylase (EC.3.2.1.1) from the thermophilic fungus *Thermomyces lanuginosus* and have studied its relation to some of its physicochemical properties. The optimum temperature of activity was 60 °C, and the optimum pH of activity was between 4.6 and 5.2. The thermostability at 60 °C was highest at pH 6.5. When exposed to temperature in the range of 50 to 80 °C, the α-amylase was found to be thermostable at 50 °C with half-lives at 60 °C of 140 min and at 70 °C of 10 min. At 80 °C the activity was nil within 5 min. The addition of Ca^{2+} had a stabilizing effect on the enzyme which could not be obtained by addition of Ba^{2+}, Mg^{2+}, or Na^{+}.

A lot of work has been carried out on thermophilic fungus *Malbranchea sulfurea.* The production of extracellular and mycelial α-glucosidases, and other amylases, by thermophilic fungus has been reported (Gautam and Gupta 1992; Gupta and Gautam 1993). Further purification and properties of an extracellular α-glucosidase from *M. sulfurea* has been also described. The α-glucosidase showed maximum activity at pH 4.8 and was stable at pH 4-5. pH optima of yeast

α-glucosidases tend to be in the range 6-5-7.0 (Kelly and Fogartyw 1983). The enzyme showed maximal activity at 60 °C. The half-life of α-glucosidase was 6 h at 55 °C, 125 min at 60 °C, and 45 min at 65 °C. The temperature optima (60 °C) resemble that of *Lipomyces starkeyi* α-glucosidase (Kelly et al. 1985).

In various other fungal α-glucosidases, the temperature optimum has been reported as 50–55 °C (Kelly and Fogartyw 1983). Costantinho et al. (1990) recorded a broad temperature optimum of about 105–115 °C for *Pyrococcus furiosus* α-glucosidase, which is the highest temperature optimum recorded for a purified enzyme. The extracellular α-glucosidase from thermophilic fungus *M. sulfurea* is distinct from other fungal α-glucosidases in its substrate specificity. Kanlayakrit et al. (1987) have reported the purification and characterization of raw-starch-digesting glucoamylase from thermophilic *Rhizomucor pusillus*. The optimal temperature and pH were 65 °C and 4.6, respectively.

Thermophilic fungi are fungi species that have a minimum temperature of growth at or above 20 °C and a maximum temperature of growth extending up to 60–62 °C. Olagoke (2014) reported a total of seven species of thermophilic fungi isolated from three locations in Ibadan, Nigeria. These were *Absidia corymbifera*, *Gilmaniella humicola*, *Talaromyces helicus*, *Chaetomium elatum*, *Chaetomium* sp., *Humicola* sp., and *Rhizomucor pusillus*, respectively. Amylases were produced by all thermophilic fungi. The amylase activities of all the fungi used were determined at pH 6.9. The peak activities for the enzyme were shown to be at 45 °C.

16.2.1.1 Applications of Amylases

Amylases [α-amylase, β-amylase, and glucoamylase (GA)] are among the most important enzymes in present-day biotechnology. The enzymes of amylase family have great importance due to its wide expanse of potential applications. The spectrum of amylase application has widened in many other fields, such as clinical, medical, and analytical chemistry. Amylases have potential application in a number of industrial processes such as in the food, textiles, paper industries, bread making, glucose and fructose syrups, detergents, fuel ethanol from starches, fruit juices, alcoholic beverages, sweeteners, digestive aid, and spot remover in dry cleaning.

16.2.2 Cellulases

Enzymatic saccharification of cellulose requires cellulase enzyme system comprising of three types of enzymes, endo-1,4-β-glucanase (CMCase), cellobiohydrolase or exoglucanases (avicelase), and β-glucosidase (cellobiase), which act synergistically in the hydrolysis of cellulose (Nizamudeen and Bajaj 2009). Fungi and bacteria both have been exploited for production of a wide variety of cellulases and hemicellulases. However, special emphasis has been given to the use of fungi

because of their capability to produce substantial amounts of cellulases and hemicellulases. It is stated that the medium used should allow easy extraction and purification of the enzymes. The ability to use low-cost agricultural residues as substrates for growth has also been emphasized (Bajaj and Abbass 2011). In addition, the fungal enzymes are often less complex than bacterial glycoside hydrolases. This enables the cloning and recombination of the genes in *E. coli*. Considering the importance of cellulases for a wide range of industrial applications, attempts have been made by several researchers to isolate microbial cellulases with desirable industrial applications, viz., high specific activity, long shelf life, thermostability, pH stability, etc. (Nizamudeen and Bajaj 2009; Bajaj et al. 2012; Gomathi et al. 2012). In order to reduce the cost of enzyme production, low-cost agro-residues have been explored as carbon and nitrogen sources for microbial production of cellulases (Nizamudeen and Bajaj 2009; Ibrahim et al. 2013; Li et al. 2009; de Castro et al. 2010; Yadav et al. 2018).

Thermophilic cellulases are key enzymes for efficient biomass degradation. Their importance arises from the fact that cellulose swells at higher temperatures, thereby becoming easier to break down. A number of thermophilic fungi have been isolated in recent years, and the cellulases produced by these eukaryotic microorganisms have been purified and characterized at both structural and functional level (Kour et al. 2019; Shukla et al. 2016).

The molecular weight of thermophilic fungal cellulases spans a wide range from 30 to 250 kDa with different carbohydrate contents (2–50%). Thermophilic fungal cellulases are active in the pH range 4.0–7.0 and have a high temperature maximum at 50–80 °C for activity. In addition, they demonstrate remarkable thermal stability and are stable at 60 °C with longer half-lives at 70, 80, and 90 °C than those other fungi (Li et al. 2011). *Trichoderma* species are considered as most suitable candidate for cellulase production and utilization in industry as compared to *Aspergillus* and *Humicola* species. However, genetically modified strains of *Aspergillus* have capability to produce relatively higher amount of cellulase (Imran et al. 2016). Thermally stable modified strains of fungi are good future prospect for cellulase production.

Romaneli et al. (1975) worked on three thermophilic cellulolytic fungi, *Chaetomium thermophile* var. coprophile, *Sporotrichum thermophile*, and *Thermoascus aurantiacus*, to determine the conditions for a high rate of cellulose degradation. The workers determined the range of temperature over which good growth occurred using a temperature gradient incubator. The optimum temperature was then established in shake flask cultures. *T. aurantiacus* had the highest optimum growth temperature range (46–51 °C), whereas *S. thermophile* had the broadest range over which good growth occurred (36–43 °C). Optimum temperatures for the three organisms, *T. aurantiacus*, *S. thermophile*, and *C. thermophile*, were 48, 40, and 40 °C, respectively.

Twenty-seven thermophilic and thermotolerant fungal strains were isolated from soil, decaying organic matter and sugarcane piles. The isolates were selected based on their ability to grow at 45 °C on medium containing corn straw and cardboard as carbon sources (Moretti et al. 2012). These fungi were identified in the genera

Aspergillus, *Thermomyces*, *Myceliophthora*, *Thermomucor*, and *Candida*. The majority of the isolated strains produced xylanase and cellulases under solid-state fermentation. Two strains, namely, *Aspergillus fumigatus* M.7.1 and *Myceliophthora thermophila* M.7.7, produced the highest amounts of cellulase and xylanase. The enzymes from these strains showed maximum activity at pH 5.0 and at 60 and 70 °C. The endoglucanase from *A. fumigatus* was stable from 40 to 60 °C, and both endoglucanase and xylanase from *M. thermophila* were stable in this temperature range in the absence of substrate. The enzymes were stable from pH 4.0 to 9.0.

Pereira JDe et al. (2015) have worked on *Myceliophthora thermophila* JCP 1–4 which produces avicelase. This enzyme is used to hydrolyze crystalline cellulose. Thirty-two heat-tolerant fungi were isolated from the environment and identified. Further the production of the enzymes was evaluated by solid-state fermentation using lignocellulosic materials as substrates. *Myceliophthora thermophila* JCP 1–4 was the best producer of endoglucanase (357.51 U g^{-1}), β-glucosidase (45.42 U g^{-1}), xylanase (931.11 U g^{-1}), and avicelase (3.58 U g^{-1}). These enzymes were most active at 55–70 °C and stable at 30–60 °C. Some cellulolytic mesophilic fungi, known for their ability to produce cellulases, as *Aspergillus* and *Trichoderma* strains, also showed low avicelase productions (Macris and Galiotou-Panayotou 1986). Such data reinforce the importance of prospecting new fungal strains and cultivation conditions to produce cellulases and xylanases, as enzymes with interesting characteristics were obtained, especially in relation to avicelase, an enzyme not commonly found among these microorganisms.

Besides, some yeasts are able to ferment xylose to ethanol, such as genetically modified *Saccharomyces cerevisiae* (Katahira et al. 2008; Kim et al. 2014; Goncalves et al. 2014; Latimer et al. 2014), *Pichia stipis* (Karimi et al. 2006; Buaban et al. 2010), and *Spathaspora passalidarum* (Long et al. 2012). In addition, some filamentous fungi are also able to ferment xylose to ethanol such as *Fusarium verticillioides* and *Acremonium zeae* (Almeida et al. 2013), *Neolentinus lepideus* (Okamoto et al. 2012), and *Trametes hirsuta* (Okamoto et al. 2011).

Nairn and Jarnil (2007) have reported an endoglucanase (endo-1,4-D-glucanase, EC 3.2.1.4) was produced from a thermostable fungus *Chaetomium thermophile*. It was grown on Vogel's medium with different carbon sources like xylan, carboxymethylcellulose, corncobs, and glucose for 5 days at 180 rpm at 28 °C in orbital shaker. Production of endoglucanase was very low with glucose as carbon source, whereas xylan and carboxymethylcellulose produced the enzymes in appreciable amount. Growth conditions of *Chaetomium thermophile* were optimized for maximal production of endoglucanase (EG): pH 5.0, temperature 50 °C, incubation period 120 h, and substrate 1% carboxymethylcellulose.

Schuerg et al. (2017) have reviewed the work on *Thermoascus aurantiacus*. Thermotolerant cellulase enzymatic mixtures from thermophilic fungi are an attractive alternative to currently available commercial cellulase cocktails. *Thermoascus aurantiacus* is a thermophilic ascomycete fungus within the order of *Eurotiales* that was first isolated by Miehe in 1907. Strains of *T. aurantiacus* have been isolated from a variety of terrestrial environments, which all have been shown to be homothallic and produce large amounts of ascospores with an optimal growth temperature

at ~50 °C. *T. aurantiacus* secretes high titers of cellulases (>1 g/L) when grown in the presence of plant biomass substrates and produces a remarkably simple cellulose mixture consisting of GH7 cellobiohydrolase, GH5 endoglucanase, AA9 lytic polysaccharide monooxygenase, and GH3 beta-glucosidase.

The production of hydrolytic enzymes by *T. aurantiacus* has been performed under solid-state fermentations using lignocellulosic materials. It was demonstrated that inoculum size of the fermentation medium influenced the production of hemicellulases and cellulases. Filtrates from the cultures were used to hydrolyze a pulp of sugarcane bagasse and enzymes produced were shown to posses good application as coadjuvants in plant saccharification (Monte et al. 2010). Interestingly, *T. aurantiacus* was cultivated on four different agricultural residues: sugarcane bagasse, sugarcane straw, wheat straw, and corncob. Xylanases and cellulases purified from filtrates of the cultures were analyzed for the hydrolysis of a bagasse pulp prepared with alkaline peroxide. Results indicated that the xylanase action on alkaline-pretreated sugarcane bagasse enhanced the cellulolytic effect promoted by a commercial cellulase. Nevertheless, this study thus presents an evaluation of the applicability of enzymes from *Thermoascus aurantiacus* to potentially improve the enzymatic cellulose hydrolysis.

T. reesei strains produce high levels of cellulases; hence, there seems to be a well-suited starting point for obtaining improved cellulose hydrolysis via boosting of coadjuvant enzymes. However, several thermophilic cellobiohydrolases of family 7 performed better than *T. reesei* cel 7A in the hydrolysis of substrates at 45 °C (Wojtczak et al. 1987).

Some filamentous fungi produce cellulases that retain relatively high cellulose-degrading activity at temperatures of 50–70 °C, particularly species such as *Thielavia terrestris*, *Aspergillus terreus*, *Sporotrichum thermophile* QM-9382, and *Thermoascus aurantiacus* (Wojtczak et al. 1987; Romaneli et al. 1975). *T. aurantiacus* is specially a good producer of β-glucosidases. The β-glucosidases of this species show high stability, half-lives at 70 °C of 23.5 h (Gomes et al. 2000), and optimum temperature and pH between 65 and 80 °C and 4.5 and 6, respectively (Gomes et al. 2000; Hong et al. 2007). Different *T. aurantiacus* strains show endoglucanases with acid pI (around 3.5–3.7) (Hong et al. 2007; Parry et al. 2001) that display high stability (at 70 °C half live of 98 h) (Gomes et al. 2000) and optimum temperature and pH between 65 and 80 °C and 4.0 and 5.5, respectively (Parry et al. 2001; Kalogeris et al. 2003). *T. aurantiacus* has also been found to produce most of the hemicellulolytic enzymes, endoxylanase being the main enzyme detected in its culture, similar to several other well-known hemicellulase-producing microorganisms such as *Aspergillus niger* (Bailey and Poutanen 1989; Kang et al. 2004; Coral et al. 2002) and *Trichoderma reesei* (Juhasz et al. 2005).

Kawamori et al. (1987) studied a thermophilic fungus, strain A-13I, isolated from a soil sample for production of cellulases in the culture medium. The fungus (strain A-13I) was identified as *Thermoascus aurantiacus* Miehe from its taxonomical characteristics. The cellulases of *T. aurantiacus* A-131 did not require a cellulose inducer, and production was constitutive in nature. Moreover, their production was induced markedly by amorphous polysaccharides containing (J-I, 4 linkages) such

as alkali-treated bagasse and xylan rather than crystalline cellulose. The cultivation of *T. aurantiacus* A-131 at 45 °C with 4% alkali-treated bagasse led to production of about 70Ujml of carboxymethylcellulase after 4 days. The thermostability of the cellulolytic enzymes of *T. aurantiacus* A-131 was excellent, and no decreases in their activities were observed after preincubation at 60 °C for 24 h.

Tong and Cole (1982) found that *Thermoascus aurantiacus* was the most active cellulase producer of several thermophilic fungi tested during their studies. Thermophilic fungi were isolated from samples of sand and decomposed woody materials collected on coastal beaches and compost heaps in Christchurch, New Zealand. The optimum growth temperature for *T. aurantiacus* in liquid medium was 45 °C, and maximum cellulose production from filter paper occurred at 40 °C. The optimum temperature for 3-glucosidase and carboxymethylcellulase activity was 70 ° C. Maximum activity was found at acidic pH, i.e., pH 5.0, for the filter paper degrading enzyme and α-glucosidase and pH 4.3 for carboxymethylcellulase activity.

Giorgi (2017) worked on fungal isolates from Georgia. From the collection of microscopic fungi isolated from ecological niches of Georgia at S. Durmishidze Institute of Biochemistry and Biotechnology of Agricultural University, thermophilic micromycetes – active producers of stable cellulases – have been selected. Four endoglucanases were purified to homogeneity from *Sporotrichum pulverulentum*, *Aspergillus wentii*, *Aspergillus versicolor*, and *Chaetomium thermophile* culture medium. Some kinetic, physical, and chemical properties of purified endoglucanases (molecular mass, isoelectric point, carbohydrates content, pH, temperature optimums, Km, Kcat, Vmax, Ki, Henries constant K_p, substrate specificity) have been studied and reported.

Bajaj et al. (2014) studied fungal isolate *Sporotrichum thermophile* LAR5 for successful utilization of low-cost agricultural residues as the substrates and production of considerable titer of cellulase. Using wheat bran as substrate maximum cellulase production (2000 IU/L) was obtained, followed by maize bran (1800 IU/L) and rice husk (1600 IU/L). Cellulase production was enhanced substantially by peptone (7900 IU/L) addition, also by mustard cake (7000 IU/L) and soybean meal (6000 IU/L) as compared to control (2000 IU/L); cotton cake and casein too supported higher enzyme production (3900 IU/L and 3800 IU/L, respectively). Though optimum temperature for cellulase activity was 60–70 °C, significant activity was observed even at higher temperatures (80–90 °C). Cellulase showed thermostability at 50–60 °C for 30 min which decreased as the time and temperature increased further.

16.2.3 Lipases

Microbial lipases have special attention industrially due to their stability toward extremes of temperature and pH and also because they have broad substrate specificity (Dutra et al. 2008; Griebeler et al. 2011; Yadav 2015). Lipases are ubiquitous

in nature and are active at different temperatures. The cold-active lipases have good activity in the temperature range of 0–30 °C (Cai et al. 2009; Yadav et al. 2017b, c). Divya and Naga Padma (2014) describe the study on cold-active lipase-producing yeasts identified morphologically and biochemically as *Geotrichum* sps and *Rhodotorula* sps. These species were isolated and tested for their ability to degrade different oils. The selected yeast isolates produced cold-active lipase at 25 °C, and the enzyme was active at 15 and 20 °C. Thus, these good cold-active lipase producers have potential industrial and environmental significance.

Berhanu and Gessesse (2012) have nicely reviewed the microbial lipases in general and their industrial applications. Thermophilic microorganisms and enzymes stable at high temperatures and adverse chemical environments are of advantage in industrial uses. One of the unique characteristics of lipases is that they remain active in organic solvents in the field of industrial application. When immobilized lipases are used under typical industrial conditions, fermentor/reactor temperatures as high 70 °C are possible for prolonged periods.

Kavitha (2016) in her review on the cold lipases have commented that cold-active lipases (CLPs) are gaining importance nowadays as they are increasingly used in fine chemical synthesis, bioremediation, and food processing and as detergent additive. These enzymes exhibit high catalytic activity at low temperatures and flexibility to act at low water medium. Since they are active at low temperatures, they consume less energy and also stabilize fragile compounds in the reaction medium. CLPs are commonly obtained from psychrophilic microorganisms which thrive in cold habitats. It is an observation that very few CLPs have been studied and used industrially as compared to the mesophilic and thermophilic lipases. CLPs (*C. antarctica* lipase-A and *C. antarctica* lipase-B) from *Candida antarctica* isolated from the Antarctic region are the well studied and industrially employed, and many are being followed up.

A cold-active lipase produced by bacteria and yeast isolates from the core sample of Nella Lake, Larsemann Hills region, East Antarctica, was investigated (Maharana and Singh 2018). Among potential yeasts and bacteria producing lipases, best isolates were identified as *Cryptococcus* sp. Y-32 and *Rhodococcus erythropolis* N149 by molecular technique. The isolate again is subjected for optimization processes using various physiological (temperature and pH) and chemical (carbon, nitrogen, minerals, and various substrates like oils and triglycerides) parameters for optimizing the lipase production capabilities. The results indicated that a supplement of 1% w/v fructose, 0.1% w/v KCl, and 2% v/v tween 80 at pH 8.5 and 15 °C enhanced the lipase production using *Rhodococcus erythropolis* N149.

Notably, the activators are 1% w/v of galactose and peptone, 0.1% w/v KCl and 2.5% v/v ghee at pH 11.5 and 15 °C. These activators enhanced the lipase production by 4.01-fold (3.35 U/ml) using *Cryptococcus* sp. Y-32. This study successfully produced cold-active lipases with novel properties like low temperature and high pH stability, which can be used in the degradation of lipid wastes in cold regions. These lipases have industrial application and can be used in detergent formulations for cold temperature washing of delicate clothes.

Divya and Padma (2015) found that among the six lipolytic yeasts, two isolates identified as *Geotrichum* and *Rhodotorula* sps were found to be good cold-active lipase producers. They were selected based on their activity to hydrolyze palm olein on a selective agar incorporated with Nile blue sulfate and palm olein. Lipases isolated from different sources have a wide range of properties depending on their sources with respect to thermostability, positional specificity, pH optimum, fatty acid specificity, etc. The selected yeast isolates showed efficient enzyme production at 25 °C and pH 7.2. The enzyme produced was active at both 15 °C and 20 °C, thus making it a potential enzyme for application in dry cleaning industry.

Lipases are widely used in the processing of fats and oils, detergents and degreasing formulations, food processing, the synthesis of fine chemicals and pharmaceuticals, paper manufacture, and production of cosmetics (Rubin and Dennis 1997). Lipase can be used to accelerate the degradation of fatty waste materials (Masse et al. 2001) and a synthetic plastic (polyurethane) (Takamoto et al. 2001).

16.2.3.1 Applications of Lipases

Fats and oils are essential constituents of foods. Lipases have also been widely used in food industry to modify flavor by synthesis of esters of short-chain fatty acids and alcohols which are known flavor and fragrance compounds (Macedo et al. 2003). Lipases are also used to remove fat from meat and fish products to produce lean meat (Kazlauskas and Bornscheur 1998). The most commercially important field of application for hydrolytic lipases is their addition to detergents which are used mainly in household and industrial laundry and in household dishwashers. Particularly, the use of cold-active lipase in the formulation of detergents would be of great advantage for cold washing that would reduce the energy consumption and wear and tear of textile fibers (Feller and Gerday 2003).

Lipases are used to remove the pitch from the pulp produced during papermaking processes (Jaeger and Reetz 1998). Nippon Paper Industries, in Japan, have developed a pitch control method that uses the *Candida rugosa* fungal lipase to hydrolyze up to 90% of the wood triglycerides (Jaeger and Reetz 1998). For bovine hides, lipases allow tensile to be completely replaced. For sheepskins, the use of solvents can also be replaced by lipases and surfactants. Lipases in organic synthesis: most of lipases used as catalysts in organic chemistry are of microbial origin. The use of lipases in the synthesis of enantiopure compounds has been reported by Berglund and Hult (2000).

Lipases can catalyze ester syntheses, and transesterification reactions in organic solvent systems have opened up the possibility of enzyme-catalyzed production of biodegradable polyesters. Lipases are widely used in the textile industry to remove size lubricants and thereby to provide a fabric with greater absorbency for improved levelness in dyeing. It is also used to reduce the frequency of streaks and cracks in the denim abrasion systems. Lipases together with alpha-amylase are used for the desizing of denim and other cotton fabrics at commercial scale (Rowe 2001).

The use of poly-esterase (closely related to lipase) can improve the ability of a polyester fabric to uptake chemical compounds, such as cationic compounds, fabric finishing compositions, dyes, antistatic compounds, anti-staining compounds, antimicrobial compounds, antiperspirant compounds, and/or deodorant compounds (Rowe 2001).

Some cosmetic industries currently produce isopropyl myristate, isopropyl palmitate, and 2-ethylhexyl palmitate for use as an emollient in personal care products such as skin and suntan creams, bath oils, etc. In this case, immobilized *Rhizomucor miehei* lipase was widely used as a biocatalyst. Lipases play an important role in modification of monoglycerides for use as emulsifiers in pharmaceutical applications (Sharma et al. 2001). Lipase from *Candida rugosa* has been used to synthesize lovastatin, a drug that lowers serum cholesterol level. *S. marcescens* lipase was widely used for the asymmetric hydrolysis of 3-phenylglycidic acid ester (Matsumae et al. 1993).

16.2.4 Proteases

Proteases are the enzymes that hydrolyze the peptide linkage of proteins into simpler proteins, peptides, and free amino acids. Unlike other enzymes, they are considered as mixture of enzymes (Lee et al. 2002) and include proteinases, peptidases, and amidases, which hydrolyze intact proteins, peptides or peptones, and amino acids, respectively. Proteases are commonly classified according to their pH: acid proteases (pH 2.0–6.0), neutral proteases (pH 7.0 or around 7.0), and alkaline proteases (pH 8–11). They are also classified on the basis of critical amino acid required for their catalytic functions (e.g., serine proteases), the chemical nature of the catalytic site (e.g., amino peptidases), or their requirement of a free thiol group (e.g., thiol proteinases) (Rao et al. 1998).

Humicola lanuginosa and *Malbranchea pulchella* were first identified to produce alkaline protease enzyme. Molds of the genera *Aspergillus*, *Penicillium*, and *Rhizopus* are mostly used for the production of industrially important alkaline protease enzyme (Devi et al. 2008). It has numerous applications in our daily life such as in food industries, bakery, wastewater refinement, medicinal formulation, detergent formulation, alcohol production, beer production, leather industries, meat tenderization, dairy industry, silver recovery, and oil manufacturing industries (Anwar and Saleemuddin 1998).

Filamentous fungi can effectively secrete various hydrolytic enzymes, and one of the main groups of secreted enzymes in fungi is protease. The proteases of *Aspergillus* species, in particular, were studied in detail. These species are known for their capacity to secrete high levels of enzymes in their growth environment. A variety of microorganisms such as bacteria, fungi, yeast, and actinomycetes are known to produce these enzymes (Madan et al. 2002).

Even though most commercial proteases originated from microorganisms belonging to the genus *Bacillus*, fungi exhibit a wider variety of proteases than

bacteria. Furthermore, fungi are normally generally regarded as safe strains, and they produce extracellular enzymes, which are easier to be recovered from fermentation broth. Molds of the genera *Aspergillus*, *Penicillium* and *Rhizopus* are especially useful for producing proteases, as several species of these genera are generally regarded as safe (Sandhya et al. 2005). Chandrasekaran and Sathiyabama (2014) recently reported a protease-producing fungus from soil. The enzyme was then produced in shake flask, and the critical production parameters like pH and temperature were optimized. Ortiz et al. (2016) did a comparative study of the proteolytic enzyme production using 12 *Aspergillus* strains using solid-state fermentation. Among these, seven strains were found to possess high and intermediate level of protease activity. From these four strains with the highest productivity, the proteolytic extract of *A. sojae* ATCC20235 was shown to be an appropriate biocatalyst for hydrolysis of casein and gelatin substrates, increasing its antioxidant activities in 35% and 125%, respectively.

In another study, two fungi *Aspergillus nidulans* and *Aspergillus glaucus* from mushroom compost and two fungi *Aspergillus terreus* and *Aspergillus fumigatus* from cow manure, showing alkaline protease activity, were isolated (Singhania et al. 2018). The zones of clearance were observed for *Aspergillus nidulans*, *Aspergillus glaucus*, *Aspergillus terreus*, and *Aspergillus fumigatus* species. The best enzyme production was observed in *Aspergillus terreus* (1.005 ± 0.057 IU/mg protein) obtained from cow manure and the minimum enzyme activity was observed with *Aspergillus glaucus* (0.278 ± 0.026 IU/mg protein). However, more studies are required to assess the potential of *Aspergillus nidulans*, *Aspergillus glaucus*, *Aspergillus terreus* and *Aspergillus fumigatus* species.

Penicillium fellutanum was isolated from mangrove sediments and was studied for production of alkaline protease in submerged fermentation (Manivannan and Kathiresan 2007). Zanphorlina et al. (2011) report the purification of a novel alkaline protease enzyme from a new thermophilic fungus *Myceliophthora* sp. The molecular weight of the enzyme was determined as 28.2 kDa by using MALDI-TOF MS, and it was inhibited by PMSF indicating it is a serine protease. The optimum pH and temperature were 9.0 and 40–45 °C, respectively.

16.2.5 Pectinases

Pectinase enzymes catalyze the breakdown of pectin, a key component of the plant cell wall. Biodegradation of pectin requires a pool of several enzymes, collectively named as pectinases. These pectinases include pectin methyl esterases, pectin acetyl esterases, polygalacturonases, polymethylgalacturonases, polygalacturonate lyases, polymethylgalacturonate lyases, rhamnogalacturonase, arabinases and xylogalacturonases (Adapa et al. 2014). At industrial level, pectinases are used in diverse applications, especially in food processing industry. Currently, most of the industrial pectinases have optimal activity at mesophilic temperatures. Pectinases have great biotechnological potential, mainly in the food industry. Pectinases are used to

remove the suspended pectin from raw juices in fruit juice processing, thus decreasing the viscosity that inabilities the filtering process. In wine making, in addition to the improvement of mash filtering, pectinases are used to improve the juice extraction from the grapes and to release compounds responsible for the color and aroma in wines (Zoecklein et al. 1997; Gang et al. 2001).

Among the microorganisms able to degrade pectin, the filamentous fungi are among the most efficient. They demonstrated a great capability of secreting a wide range of pectin-degrading enzymes, and currently, most of the commercial pectinolytic enzymes available are produced by filamentous fungi, particularly from genera *Aspergillus*, *Trichoderma*, and *Penicillium* (Benoit et al. 2012; Gupta 2011; Lara-Márquez et al. 2011). On the contrary, very little is known about the pectinolytic activities from organisms from cold climates such as Antarctica. These mesophilic commercial pectinases posssess an optimal temperatures between 40 and 60 °C (Adapa et al. 2014). However, there are processes where pectin degradation is necessary at lower temperatures. For example, the clarification of the mash for the production of white wine and pisco is performed at 15 °C. This low temperature is required to avoid the propagation of microbiota and to keep the aromatic molecules intact, which confer the organoleptic characteristics to these products. Recently Reynolds et al. (2018) in their investigations indicated that commercial pectinases with mesophilic characteristics do not work efficiently during wine fermentations at low temperatures. Thus, the interest to seek cold-active pectinases has been increasing. These cold-active pectinases potentially could replace the existing mesophilic commercial enzymes in low-temperature processes.

Poveda et al. (2018) in their work isolated 27 filamentous fungi from marine sponges collected in King George Island, Antarctica. These were screened as new source of cold-active pectinases. Eight out of 27 of these isolates showed pectinolytic activities at 15 °C, and *Geomyces* sp. strain F09-T3–2 showed the highest production of pectinases in liquid medium containing pectin as sole carbon source. More interesting, *Geomyces* sp. F09-T3–2 showed optimal pectinolytic activity at 30 °C, 10 °C under the temperature of currently available commercial mesophilic pectinases. Thus, pectinases from filamentous fungi with optimal activity lower than 40 °C were identified only in *Botrytis cinerea* (Pan et al. 2014).

Sahay et al. (2013) studied the cold-active pectinolytic enzymes (PME, endo-PG, and exo-PG) from the newly isolated and identified psychrophilic yeast *Cystofilobasidium capitatum* SPY11 and psychrotolerant yeast *Rhodotorula mucilaginosa* PT1 that exhibited 50–80% of their optimum activity under some major oenological conditions pH (3–5), and temperatures (6 and 12 °C) could be applied to wine production and juice clarification at low temperature. Of the 23 morphotypes of yeasts capable of utilizing pectin as sole carbon source at 6 °C that were isolated from soil, 2 yeast isolates, 1 psychrotolerant (PT1), and 1 psychrophilic (SPY11) were selected according to their ability to secrete pectinolytic enzymes under some oenological conditions (temperature 6 and 12 °C and pH 3–5) and ability or inability to grow above 20 °C, respectively. The psychrotrophic yeasts themselves could be applied to cold process for the production of enzymes thus saving cost of energy and protecting process from contamination.

As most strains of *Saccharomyces cerevisiae*, used in wine industries, do not show pectinolytic activity (Merín et al. 2011), non-wine yeasts was explored as source of these enzymes (Strauss et al. 2001). The use of non-wine yeast may result in contamination or may result in the production of undesirable metabolic products. Application of enzymes therefore is a better alternative to avoid these problems possessed better control over process and quality of end product. According to the authors, this study was designated to isolate psychrotrophic yeasts capable of secreting cold-active pectinolytic enzymes (PME, endo-PG and exo-PG) having potential for the application in wine making.

16.2.6 Xylanases

Xylanase productions obtained in the present study are similar to some cited in scientific literature regarding thermophilic fungi cultivation by SSF. Monte et al. (2010) reported xylanase productions of 1315.9, 978.0 and 1679.8 U g^{-1} when cultivating *Thermoascus aurantiacus* on wheat bran, sugarcane bagasse, or sugarcane straw, respectively. A yield of 1292.0 U g^{-1} of xylanase by *M. thermophila* M_7_7 was cited by Moretti et al. (2012), when using a mixture of sugarcane bagasse and wheat bran as substrates. *Aspergillus fumigatus* P40M2, when cultivated on wheat bran, showed a xylanase production of 1055_62 U g^{-1} (Delabona et al. 2013).

Bergquist et al. (2002) used yeast *Kluyveromyces lactis* and the filamentous fungus *Trichoderma reesei* for the extracellular production of thermophilic enzymes for the pulp and paper industry. The *K. lactis* system has been tested with two thermophilic xylanases and secretes gram amounts of largely pure xylanase A from *Dictyoglomus thermophilum* in chemostat culture. *T. reesei* expression system was developed involving the use of the cellobiohydrolase I (CBHI) promoter and gene fusions for the secretion of heterologous thermostable xylanases of both bacterial and fungal origin. A heterologous fungal gene, *Humicola grisea* xyn2, could be expressed without codon modification.

Yu et al. (1987) screened 21 strains of thermophilic fungi in the Forintek culture collection xylanolytic (and cellulolytic) enzyme production in both solid and aqueous media containing various hemicellulosic and cellulosic substrates. *Thermoascus aurantiacus* strain C436 was selected as the best producer of extracellular xylanase (1,4-β-D-xylan xylanohydrolase, EC 3.2.1.8) enzymes. High xylanase activity was detected in fungal culture filtrates using lignocellulosic residues such as steam-exploded aspenwood and untreated aspenwood sawdust as substrates. Maximum xylanase activity (575.9 U ml^{-1}) was detected in cultures grown in Vogel's medium containing oat-spelt xylan. The xylanase activity exhibited a temperature optimum of 75 °C and pH optimum around 5.0. The half-lives of the xylanase activity at 70 and 60 °C were 1.5 h and 4 days, respectively. Crude culture filtrates concentrated by membrane ultrafiltration could effectively hydrolyze xylan and steam-exploded aspenwood hemicellulose to release near theoretical yields of low molecular weight pentose oligomers.

Bergquist et al. (2002) reported developing the yeast *Kluyveromyces lactis* and the filamentous fungus *Trichoderma reesei* for the extracellular production of thermophilic enzymes for the pulp and paper industry. A cDNA gene encoding a family 11 xylanase (xyn2) was isolated recently from *Humicola grisea* var. thermoidea cultivated from Brazilian soil. The *H. grísea* XYN2 gene product is highly active at 70 °C and pH 6.5 (Faria et al. unpublished). The xyn2 gene was cloned into *T. reesei* as a fusion to the cbhl signal sequence and the CBHI core-linker. The recombinant XYN2 yields were of the order of 0.5–1 g per liter in non-optimized shake flask cultivations, and activities up to about 50,000 nkat/ml was obtained by small-scale fermentation. Brienzo et al. (2008) worked on different strains of the thermophilic ascomycetous fungus *Thermoascus aurantiacus.* They reported production of high levels of a variety of enzymes of industrial interest (i.e., amylases, cellulases, pectinases, and xylanases), which were remarkably stable over a wide range of temperatures. Most studies on enzyme production by *T. aurantiacus* were carried out in chemically defined liquid medium, under conditions suitable for induction of a particular enzyme. A few studies investigated the production of some enzymes by *T. aurantiacus* by solid-state fermentation, using lignocellulosic materials. The authors primarily focused on the enzymes produced by *T. aurantiacus*, their main kinetic parameters, and the effect of different culture conditions on production and enzyme activity. The possible applications of *T. aurantiacus* enzymes also been touched upon, considering that this thermophilic fungus could comprise a potential source of thermostable enzymes. Joshi and Khare (2012) investigated the regulation of xylanase production in two thermophilic fungi *Scytalidium thermophilum* and *Sporotrichum thermophile*. Various carbon sources were used for induction of the enzyme. Soy flour and oat-spelt xylan induced maximum level of xylanase in *Scytalidium thermophilum* and *Sporotrichum thermophile*, respectively. Induction of xylanase in *Scytalidium thermophilum* led to simultaneous induction of cellulase. Basit et al. (2018) describe cloning of two new GH11 xylanase genes, MYCTH_56237 and MYCTH_49824, from thermophilic fungus *Myceliophthora thermophila* and expressed in *Pichia pastoris*. The specific activities of purified xylanases reach approximately 1533.7 and 1412.5 U/mg, respectively. Enzyme activity was more effective in 7.5 L fermentor, yielding 2010.4 and 2004.2 U/mL, respectively. Both enzymes exhibit optimal activity at 60 °C with pH of 6.0 and 7.0, respectively. Xylan is the principal type of hemicellulose. It is a linear polymer of β-D-xylopyranosyl units linked by (1–4) glycosidic bonds.

An enzymatic complex is responsible for the hydrolysis of xylan, but the main enzymes involved are endo-1,4-β-xylanase and β-xylosidase. These enzymes are produced by fungi, bacteria, yeast, marine algae, protozoans, snails, crustaceans, insect, seeds, etc., but the principal commercial source is filamentous fungi. Recently, large focus has been laid on industrial xylan and its hydrolytic enzymatic complex, as a supplement in animal feed, for the manufacture of bread, food and drinks, and textiles, bleaching of cellulose pulp, and ethanol and xylitol production. This review describes some properties of xylan and its metabolism, as well as the biochemical properties of xylanases and their commercial applications (Polizeli et al. 2005).

Robledo et al. (2016) report on the thermophilic fungal strains able to grow at high temperatures (≥55 °C) and which were isolated from maize silage. The strains were used for the production of extracellular xylanase by solid-state fermentation using corncobs as support-substrate material. Species from the genera *Rhizomucor* and *Aspergillus* were identified among the isolated strains, and these species demonstrated good ability to produce xylanase under solid-state fermentation conditions. The xylanase produced by this fungus presented thermal stability at 75 °C, with maximum activity at 70 °C and pH 6.0, revealing, therefore, great potential for application in different areas.

Twenty-seven thermophilic and thermotolerant fungal strains were isolated from soil, decaying organic matter, and sugarcane piles based on their ability to grow at 45 °C on medium containing corn straw and cardboard as carbon sources (Moretti et al. 2012). These fungi were identified in the genera *Aspergillus*, *Thermomyces*, *Myceliophthora*, *Thermomucor*, and *Candida.* The majority of the isolated strains produced xylanase and cellulases under solid-state fermentation. The highest cellulase and xylanase productions were obtained by the cultivation of the strains identified as *Aspergillus fumigatus* M.7.1 and *Myceliophthora thermophila* M.7.7. The enzymes from these strains exhibited maximum activity at pH 5.0 and at 60 and 70 °C. The endoglucanase from *A. fumigatus* was steady from 40 to 65 °C, and both endoglucanase and endoxylanase from *M. thermophila* were steady in this temperature range.

Ramanjaneyulu et al. (2015) reported the isolation of 450 fungal cultures from forest soils of Eastern Ghats of Andhra Pradesh, India, and screened the isolates by Congo red plate assay. Xylanase activity of eight best producers was assessment in submerged fermentation (SmF) by assessing the amount of reducing sugar released by using 3,5-dinitrosalicylic acid (DNS) method. Ahmed et al. (2012) reported findings on partially purified xylanases preparation from *T. harzianum* and *C. thermophilum*. They found that the enzyme preparations exhibited optimal activities at pH 5 and pH 6 and at 60 and 70 °C, respectively. The apparent Km and Vmax values for the partially purified xylanase from *T. harzianum* using oat-spelt xylan as a substrate were 4.8 mg mL-1 and 0.526 μmol min-1 mg-1, respectively. These findings in this study have great implications for future applications of xylanases.

A study on isoforms of xylanases of 13 fungi was reported by Ghatora et al. (2006). They worked on the isoforms of xylanases produced by these thermophilic fungi. Eighty-three xylanases were found to be produced by these fungi, by isoelectric focusing. Among these thermophiles, four species, namely *Chaetomium thermophilum*, *Humicola insolens*, *Melanocarpus* sp., *Malbranchea* sp., and *Thermoascus aurantiacus*, produced alkaline active xylanases. An enzymatic complex is responsible for the hydrolysis of xylan, but the main enzymes involved are endo-1,4-β-xylanase and β-xylosidase. Abdelrahim and Bayoumi (2011) described their research work which is mainly focused on four strains of thermophilic fungi, viz., *Sporotrichum thermophile*, *Chaetomium thermophile*, *Humicola grisea*, and *Torula thermophila.* The fungi were screened for their production of xylanolytic enzymes in soluble and lignocellulosic insoluble substrate including Kallar grass, xylan, glucose, cellobiose, and wheat bran. Studies using Kallar grass as single substrate for enzyme production were carried out, and it was observed that when supplemented with 0.5% xylan, the enzyme activity was found to increase. The

carbon source combination consisting of Kallar grass plus xylan plus glucose was found to be the best for production of xylanases from these fungi in order of *H. grisea*, *C. thermophila*, *T. thermophila*, and *S. thermophila*. The optimum temperature for xylanase assay produced from the various species of fungi was found to be 70 °C. Notably, the four crude enzymes were deemed as potential candidates in feed and food industry applications.

Nitin et al. (2017) studied the production of xylanase enzyme (EC number = 3.2.1.8) using solid substrate fermentation. Optimum xylanase activity was observed when pea peel has been used as solid substrate. Agro-industrial wastes are a good source of nutrition for the growth of the microorganisms as they are rich in carbon source, and agro-industrial wastes such as wheat bran, sugarcane bagasse, corncob, rice bran, and wheat straw are abundantly available and cheapest natural carbon sources. The present study was an attempt for process optimization for xylanase production using agro-industrial waste as a sole carbon source. Different physical and chemical parameters which affect the production of xylanase were optimized by batch experiment as well as using statistical tool, i.e., Design-Expert. This work showed that agro-industrial residue has excellent potential for the production of industrial important enzyme, i.e., xylanase.

Maheshwari and Kamalam (1985) described an uncommon thermophilic fungus, *Melanocarpus albomyces*. It was isolated from soil and compost. High extracellular xylanase (EC 3.2.1 0.8) activity was produced by culture and was grown on xylose or hemicellulosic materials. Gel-filtration chromatography of culture filtrate protein showed the presence of two isoenzymes of xylanase, whose relative proportions varied with the carbon source used for growth. The use of culture filtrate protein preparations of cultures grown on bagasse showed greater extent of hydrolysis of heteroxylans or the hemicellulosic fraction of bagasse than that of cultures grown on xylose as the inducing substrate. Azad et al. (2013) described two thermostable xylanase-producing thermophilic fungi, *Thermomyces lanuginosus* BPJ-10 and *Rhizomucor pusillus* BPJ-2. When grown under solid-state fermentation using wheat bran, optimum production of xylanase was found to be after 4 days and 7 days for *R. pusillus* BPJ-2 and *T. lanuginosus* BPJ-10, respectively. The optimum temperatures for the production of xylanase by *R. pusillus* BPJ-2 and *T. lanuginosus* BPJ-10 were 45 and 50 °C, respectively. The maximum activity of xylanase (1.685 IU/ml and 0.075 IU/ml) was exhibited by *T. lanuginosus* BPJ-10 and *R. pusillus* BPJ-2 at pH 7.0 and pH 4.0, respectively.

Recently, there has been much industrial interest in xylan and its hydrolytic enzymatic complex, as a supplement in animal feed, for the manufacture of bread, food and drinks, and textiles, bleaching of cellulose pulp, and ethanol and xylitol production. Torre and Kadowaki (2017) explained the biochemical properties and industrial applications of xylanases in their review. The applications of filamentous fungi are in different industrial sectors, such as bakery, beverage, biofuel, textile, animal feed, pharmaceutical, pulp, and paper. The mechanisms of adaptation of thermophilic organisms to tolerate in high-temperature environments are also touched upon. These enzymatic properties of thermal and pH stability are crucial, especially in processes such as the manufacture of animal feed, pulp, and paper industry.

16.3 Biotechnological Applications of Extremophilic Enzymes

Special interest attracts thermophilic/thermotolerant fungi having potential of growth above 20 °C and optimum of growth 40–50 °C and very rare at 55–65 °C. Fungi thermophiles represent heterogeneous physiological group of various genera in the *Phycomycetes*, *Fungi Imperfecti*, *Ascomycetes*, and *Mycelia sterilia* (Maheshwari et al. 2000). Screening of microscopic fungi strains of the culture collection according to their ability to produce stable and active extracellular enzymes has been carried. Around 400 strains of genera *Aspergillus*, *Chaetomium*, *Cladosporium*, *Fusarium*, *Helminthosporium*, *Mucor*, *Penicillium*, *Rhizopus*, *Trichoderma*, *Trichothecium*, *Myrothecium*, *Penicillium*, *Stachybotrys*, and *Sporotrichum* were studied, and 48 microscopic fungi enzyme producer strains were selected. These had high activities of cellulose, xylanase, and laccase (Kvesıtadze et al. 2017). Similarly Kvesitadze et al. (2013) reported the screening of extreme thermophilic strains of fungi for their cellulose-degrading enzymes according to heat stability and salient physical-chemical characteristics. Four endoglucanases were purified to homogeneity from *Sporotrichum pulverulentum* J-3, *Aspergillus wentii* S-6, *Aspergillus versicolor* D-3, and *Chaetomium thermophile* P-21. Low-temperature active endoglucanases were obtained by several fungal strains from the marine sponge *Haliclona simulans* in Ireland (Baker et al. 2010).

The polyextremophilic behavior shown by α-amylase obtained from *Engyodontium album* TISTR 3645 makes this enzyme a suitable choice to be used in extreme conditions of industries, particularly as additive in detergents as well as for the treatment of extreme saline wastewater (Ali et al. 2014). Halophilic fungi may promise greater advantages over using bacterial counterparts in related industries due to their primary and secondary metabolites from halophilic fungi demonstrating polyextremophilic behavior. The finding of extremely thermostable starch-hydrolyzing enzymes such as amylases and pullulanases that are active under similar conditions will significantly improve the industrial starch bioconversion process, i.e., liquefaction, saccharification, and isomerization (Niehaus á et al. 1999).

Glucoamylases are typical fungal enzymes and are among the most important industrial enzymes used for the production of glucose syrups. For the saccharification of dextrin, the glucoamylases from *Aspergillus niger* and *Aspergillus oryzae* are generally used (Antranikian 1992). Chitinases active at low temperatures (5 and 10 °C) were also reported by Fenice et al. (1998) and Velmurugan et al. (2011). *Aspergillus ustus* isolated from deep-sea calcareous substrates has been reported to produce protease which was active under elevated hydrostatic pressure and low temperature. The culture *Aspergillus ustus*, NIOCC 20, showed highest amount of protease production active at alkaline pH and low temperature. The growth conditions showed substantial growth at 7.32, 5 °C, under 50 and 100 bar pressure (corresponding to 1000 m) (Raghukumar and Raghukumar 1998). An alkalophilic strain of *Streptomyces albidoflavus* has been reported to produce extracellular proteases. This enzyme hydrolyzes keratin at highly alkaline pH 10.5, under static conditions.

This enzyme is unique for its activity and stability in neutral and alkaline conditions. The maximum activity has been obtained at pH 9.0 and in the temperature range of 60–70 °C. An enhanced (sixfold) protease production could be achieved with modified composition of culture-medium containing inducer at the concentration of 0.8% in the fermentation medium. The application of this type of protease, i.e., keratinase- hydrolyzing keratins, in industries is of significance due to its tolerance to the detergents and solvents (Indhuja et al. 2012).

The thermophilic microorganism *Humicola* sps. has been studied for its capability of biosynthesizing an alkali-tolerant β-mannase xylanase, with potential applications in brewing industry (Mamo et al. 2009; Luo et al. 2012; Du et al. 2013). Acidophilic xylanases stable under acidic conditions of reaction were reported to be produced by an acidophilic fungus *Bispora* (Luo et al. 2009). Alkaline xylanases and thermostable metal-tolerant laccases were produced by marine-derived strains of *Aspergillus niger* and *Cerrena unicolor* (Raghukumar et al. 2004a, b; D'Souza-Ticlo et al. 2009). Thermophilic laccase enzyme is of particular use in the pulping industry. Forms of laccase with unusual properties were isolated from the basidiomycete culture of *Steccherinum ochraceum* (Chernykh et al. 2008) and *Polyporus versicolor* (Nigam and Prabhu 1986).

Cold-active microbial enzymes attracted increasing attention in recent years (Wang et al. 2012). These enzymes are preferred to the mesophilic and thermophilic counterparts due to the decrease in energy expenditure and processing costs associated with industrial heating steps (Duarte et al. 2013). Del-Cid et al. (2014) reported a cold-active xylanase produced by a marine-derived *Cladosporium* sp. While working on a recombinant marine fungal strain, a psychrotrophic fungus from the Yellow Sea has been reported by Hou et al. (2006).

Lipases, proteases, and cellulases were reported to be produced on solid media at 15°C by Antarctic marine yeast strains isolated from marine samples (Duarte et al. 2013). The better capacity of marine-derived basidiomycetes to decolorize and degrade textile dyes corroborates the results of many studies cited in literature using terrestrial basidiomycete fungi. The best producers of ligninolytic enzymes are the white-rot fungi. Sponge-derived basidiomycetes showed the ability to decolorize textile dyes in solid medium under both saline and non-saline conditions (Bonugli-Santos et al. 2012). In another study, by Da Silva et al. (2008), four fungi *Penicillium citrinum* CBMAI 853, *A. sulphureus* CBMAI849, *Cladosporium cladosporioides* CBMAI857, and *Trichoderma* sp. CBMAI 852 were shown to decolorize RBBR efficiently. Raghukumar et al. (2004a), demonstrated that marine-derived fungi are often more effective than terrestrial fungi in the treatment of various colored effluents because they are better adapted to perform under extreme conditions (high salinity).

Raghukumar et al. (2004b) showed efficient lignin mineralization by the basidiomycete fungus NIOCC#312 isolated from decaying sea grass. Ligninolytic enzymes present important biotechnological properties, since they might be able to degrade a wide variety of substrates via free radical-mediated oxidizing reactions. These enzymes can also be considered a great resource in the biofuel field, due to the possible resistance and activity in the presence of solvents and different pH

conditions. Intriago (2012) reported the prospect of utilizing marine microorganisms in cellulosic ethanol production. Passarini et al. (2011) evidenced that the fungus *A. sclerotiorum* CBMAI 849 and *Mucor racemosus* CBMAI847 with polycyclic aromatic hydrocarbon (PAH) degradation ability, suggesting that the mechanism of hydroxylation is mediated by a cytochrome P-450 monooxygenase. In the study performed by Wu et al. (2009), *Aspergillus* sp. BAP14 isolated from marine sediment of China coast showed the ability to degrade benzo[a]pyrene. In another study, two non-identified marine-derived fungi (NIOCC#312 and NIOCC#2a) were able to remove phenanthrene from a culture medium by adsorption on the fungal mycelium (Raghukumar et al. 2006). Considering that the use of marine-derived fungi for the bioremediation of polluted saline environments is facilitated by their tolerance to saline conditions, these microorganisms are important microbial resources for biotechnological application in the bioremediation of PAH-polluted environments, such as ocean and marine sediments.

Microbial communities in marine environments are ecologically relevant as intermediaries of energy and play an important role in nutrient regeneration cycles as decomposers of dead and decaying organic matter. In this sense, marine-derived fungi can be considered as a source of enzymes of industrial and/or environmental interest. Different enzymes produced by marine-derived fungi reported in the literature and are related to the industrial production of lipases in cosmetics, medicine, clinical reagents (Zhang and Kim 2010; Murray et al. 2013); proteases for digestive and anti-inflammatory drugs production (Zhang and Kim 2010); ligninases in industries as the chemical, fuel, food, agricultural, paper, textile, and cosmetic (Raghukumar et al. 1994; Sette and Bonugli-Santos 2013); and L-glutaminase, tannase, and alginase having potential application in the pharmaceutical and food/beverage industries (Velmurugan and Lee 2012). The potential ability of marine-derived fungi to grow on relatively rather simple and inexpensive substrates and produce enzymes with different physiological characteristics can place them at the forefront of contemporary commercial applications.

16.4 Conclusion and Future Prospects

The extremophilic enzymes play a pivotal role in several industries including detergent, leather processing, food, medical purpose, chemical industry, and so on. This range of applications is likely to increase severalfolds in the near future so as to overcome the disadvantages of chemical methods and the non-extreme enzymes. Certain marine-derived fungal strains present enzymes with alkaline and scold activity characteristics, and salinity is considered an important condition in screening and production processes. The market for marine fungal enzymes is divided into four segments: (i) technical enzymes, mainly intended for cleaning, textile, leather, biofuel, pulp, and paper industries; (ii) enzymes for food and beverages; (iii) enzymes for animal feed; (iv) enzymes related to environmental applications; and (v) enzymes related to pharmaceutical and cosmetic applications (Debashish et al.

2005). Due to their immense genetic and biochemical diversity, extremophilic fungi can be viewed as a new promising source of enzymes with potential technological applications. Further improvement in the production can be obtained by biotechnological advancements. There is a scope for the application of enzymes such as proteases in clinical applications.

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Conflict of Interest We declare that we do not have any conflict of interest.

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Chapter 17
Global Scenario of Fungal White Biotechnology: Past, Present, and Future

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Abstract White fungal biotechnology is an emerging field in scientific arena that supports revealing of novel and vital biotechnological components. Fungi used are divided in five major economically important fields such as drug manufacturing, food and dietary, environmental, agriculture and biotechnology area. "Penicillin" drug discovery from *Penicillium* fungal sp. turns into a keystone for white fungal biotechnology. Fungi are treasure island for production of various intracellular enzymes and microbial based industrial product, i.e., lead bioactive compound for drug discovery, dairy product, detergent, lignocellulose, textile and biofuel. Fungi are highly diversified group of microorganism, i.e., *Fusarium*, *Aspergillus*, *Trichoderma*, mycorrhizal fungi that produce various enzymatically active compounds, laccase, protease, chitinase, lignocellulose, etc. These heterotrophs are dominant decomposers of the soil ecosystem which allows degrading organic material, by processing the matter through biodegradation and biosorption methods. Fungi possess a symbiotic relationship with host plant based on mutualism. Fungi play an important role in plant growth promotion by producing plant growth-promoting factors, enhance phytohormone production, and secrete immune stimulatory elements. In agriculture field, crop plants are susceptible to pathogenic microbial consortia during the harvesting season, and mycorrhizal fungi play a vital role in biocontrol and also minimize abiotic stress in plants. Biofuel production using fungi is a new renewable approach to overcome fuel crisis in the world. Microbial based cleaning products replace chemicals due to high price and toxicity caused to the environment. Fungal species are versatile tools for manufacturing secondary metabolite for drug discovery and can be used as genetic model organism for insulin production. Fungi utilization can be exploited as an alternative and contemporary tactic to minimize greenhouse gas emission in the environment. Synergistic action and kinetic expression profile of saprophytic fungi are needed to explore a novel range of catalytic products that can be useful in lignocellulose degradation for synthesis of industrially important by-product. Genome data mining,

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metabolomics, proteomics, and transcriptomics can be applied to build novel scientific agenda for several unknown enzymes, genes and metabolic pathways in white fungal biotechnology.

17.1 Introduction

Microorganisms are tiny microscopic organism, unicellular or multicellular, useful for human health and society and can be dangerous at the same time. Microorganisms are beneficial to human society and categorized based on their usage such as the production of biologically important molecules in industrial or medical field, fulfilling the gap of food crisis and water treatment. Microbes can be utilized as biological weapon in bioterrorism to kill the entire army. However, there are some significant benefits of using microbes as genetic model as they live in majority of the soil ecosystem and prerequisite gut flora in humans. Human race in science field have discovered novel microbial continuum which is highly important to identify unique bioactive compound and lead molecules.

In 1872, Johannes Reinke hypothesized consortium theory which explains existence of different microorganisms, i.e., bacteria, fungi, and actinomycetes, in symbiotic relationship in a specific ecological niche. Fungi are multicellular, eukaryotic microorganisms present in endosymbiotic or echo-symbiotic relationship with the host or can be a causative agent of fungal infections. Blackwell and Vega (2018) explained the presence of microbial variety in soil ecosystem, and abundance of fungal microbial consortia was higher than the other microbial species. Symbiotic relationship between fungi and host may provide beneficial accommodation to the microbes in exchange for nutrient and protection against pathogenic microbes. Fungi are a rich source of bioactive compounds which may be useful for human in the form of nutritions, degradation of plant organic material, and detoxification of toxic compounds. Abundance of fungal species is highly affected by environmental conditions such as soil pH, soil humidity and temperature and climate change. Pieterse et al. (2018) documented the number of fungi present in *Aizoaceae* plants at Succulent Karoo biodiversity hotspot, South Africa. They found that the presence of *Fusarium* genus was high during flowering season; *Alternaria* and *Cladosporium* were higher in dry season compared to other genera *Periconia*, *Preussia*, *Talaromyces pinophilus*, *Schizothecium*, *Truncatella*, *Neophaeosphaeria*, *Fusarium oxysporum* and *Paecilomyces victoria*.

17.2 Biological Potential of Fungi

As consequences of highly diversified fungi existence in the ecosystem, scientists have identified various prospective ways to utilize the fungal biomass at commercial level. Fungal metabolites and fungal biomass are majorly used in the industrial and medical field. Here in the chapter, we have tried to explain the utmost arena where fungi can be exploited maximum for human benefit.

17.2.1 Drug Discovery

17.2.1.1 Antimicrobial Activity

Antibiotic resistance is a major threat for scientific world as it occupies half of the infectious disease causing microbes, which are evolving resistance against the conventional drug molecules. Antibiotic resistance developed by pathogenic microorganisms has put an alarm on world's scientists to discover new antimicrobials from natural sources. Microbial sources are highly rich in bioactive compounds which can be used as antibacterial and antifungal agents against pathogenic microbes. Endophytic fungi produce secondary metabolites which act as therapeutic agents to inhibit pathogenic microbial growth and to minimize the pathogenicity. Bioactive compounds are dissimilar at their structural level and are defined to perform specific activity against microbes.

Ribeiro et al. (2018) isolated 86 endophytic fungi from the stem and leaves of *Oryctanthus alveolatus* (mistletoe) and evaluated for their antimicrobial activity. Among 86 endophytic fungi, crude extract of *Curvularia* sp. (COA 009) and *Diaporthe* sp. (COA 014) showed higher antibacterial activity against *Pseudomonas aeruginosa*, *Escherichia coli*, *Staphylococcus aureus*, and *Staphylococcus epidermis* in a dose-dependent manner ranging from 100 to 1000 μg/ml concentration using disk diffusion method. Endophytic *Massarinaceae* family produces bioactive compounds, dihydrobenzofurans and xanthenes, which are effective against *Microbotryum violaceum* and *Bacillus subtilis* (Richardson et al. 2015).

Supaphon et al. (2013) isolated endophytic fungi, *Hypocreales* sp. PSU-ES26, *Trichoderma* spp. PSU-ES8 and PSU-ES38, *Penicillium* sp. PSU-ES43, *Fusarium* sp. PSU-ES73, *Stephanonectria* sp. PSU-ES172, and an unidentified endophyte PSU-ES190 from seagrass species: *Cymodocea serrulata*, *Halophila ovalis,* and *Thalassia hemprichii*. At the MIC concentration of 10 μg/ml, selected endophytic fungi showed higher antimicrobial activity against human pathogens, *S. aureus* ATCC 25923, methicillin-resistant *S. aureus*, *E. coli* ATCC 25923, *P. aeruginosa* ATCC 27853, *Candida albicans* ATCC 90028, *C. albicans* NCPF 3153, *Cryptococcus neoformans* ATCC 90112, *C. neoformans* ATCC 90113, *Microsporum gypseum,* and *Penicillium marneffei*. Supaphon et al. (2018) identified different groups of endophytic fungi from various parts of aquatic flowering plant, *Nymphaea lotus* and *Nymphaea stellate*. Among 210 fungal isolates, 195 isolates were evaluated for their antifungal activity against *Cryptococcus neoformans* and *Talaromyces marneffei*. *Eupenicillium levitum* FNL036 secreted bioactive molecules were found to be nontoxic to the human embryonic kidney 293 (293 T) and human keratinocyte (HaCAT) cell lines and cause morphological changes in the pathogenic strains. Bioactive compounds protocatechuic acid, indole-3-acetic acid, and acropyrone produced by endophytic fungus CJ-MR2, isolated from *Citrus jambhiri* (Rutaceae), showed antibacterial and antifungal activity towards *S. aureus*, *C. albicans*, and *Aspergillus fumigatus*, respectively (Eze et al. 2018). *Aspergillus oryzae* (Koji) produces kojic acid, a secondary metabolite produced as by-product during fermentation, and possesses antibacterial and antifungal properties.

Nurunnabi et al. (2018) retrieved kojic acid from *Colletotrichum gloeosporioides* isolated from mangrove *Sonneratia apetala* using HPLC analysis. At the concentration ranging from 0.125 μg/ml to 1 mg/ml, kojic acid showed antibacterial activity toward *P. aeruginosa* and *Micrococcus luteus*. Suppression of tomato seedling disease "bacterial wilt" caused by *Ralstonia solanacearum* was achieved by applying antimicrobial compound isolated from *Simplicillium lamellicola* BCP. Antimicrobial compound was characterized using spectral analysis and chemical degradation techniques, known as massoia lactone, (3R, 5R)-3-hydroxydecan-5-olide, halymecins F, halymecins G, and (3R,5R)-3-O-β-D-mannosyl-3,5-dihydrodecanoic acid. These novel compounds possess higher antimicrobial activity against *Agrobacterium tumefaciens* (Dang et al. 2014).

Wang et al. (2017a) isolated novel antimicrobial compound, anthraquinone, 2-(dimethoxymethyl)-1-hydroxyanthracene- 9,10-dione, from *Aspergillus versicolor* and docked with receptor topoisomerase IV and AmpC β-lactamase to determine the interaction and affinity to bind for inhibition of the specific receptor. The docking studies showed lowest docking score with binding energy of −4.42 kcal/mol and −4.45 kcal/mol with receptor, topoisomerase IV and AmpC β-lactamase, respectively. Hydrogen bonding and Pi-Pi interaction showed possibility of great ligand-receptor interaction supported by the docking score. The anthraquinone showed antimicrobial activity against methicillin-resistant *S. aureus* and *Vibrio campbellii*. Antimicrobial compounds generated from fungal sources are promising leads to combat the antibiotic resistance microorganism.

17.2.1.2 Antioxidant/Antitumor/Anticancer Activity

Production of free reactive oxygen species (ROS) leads to a problematic situation in cells and impaired cell metabolism. Generation of free ROS supports indefinite growth of abnormal cells and further converts into tumor or group of cancerous cells. Mutation in normal oncogene leads to the generation of mutated cells causing spread of cancer in the organs and circulatory system. Microbial bioactive compounds can suppress ROS generation and prevent cells from the side effects. These bioactive molecules exhibit antioxidant activity by enhancing the catalytic activity of superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GTH) enzymes and promote lipid peroxidation inhibition. The mechanism of action behind antioxidant properties is regulated through scavenging of free reactive oxygen species and chelation of ferrous ions and reducing the energy transportation. Camptothecin from *Camptotheca acuminate* and paclitaxel from *Taxus* sp. showed an effective alternative for chemotherapy to treat the cancer and leukemia. Endophytic fungi are able to produce variety of secondary metabolites, i.e., terpenes, quinones, steroids, isocoumarin, and alkaloids, which helps in preventing the cancer and tumor formation in human organs (Li et al. 2018).

Scientists have identified a novel endolichenic fungus EL002332 isolated from *Endocarpon pusillum*. myC is a bioactive compound isolated from the fungi and

analyzed for its cytotoxicity on AGS human gastric cancer cells and CT26 mouse colon cancer cells. Morphological changes were observed in the cancer cells due to the activation of cell apoptosis via Bcl2 family protein expression regulation and caspase pathways (Yang et al. 2018). Minarni et al. (2017) isolated *Phomopsis* sp. from *Annona muricata* L. and evaluated for cytotoxicity against MCF-7 (Michigan Cancer Foundation-7) cells by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. *Phomopsis* sp. exhibited anticancer activity at 19.20 μg/ml concentration without affecting cell proliferation rate in the medium. Pei et al. (2015) examined novel bioactive compound recovered from *Phenllinus linteus* myecelia for antitumor activity against A-549, Bel7402, HCT-8, and HepG2 cells. HepG2 cells were found more susceptible to isolated polysaccharide, the chemical structure revealed the presence of the backbone made up of (1 → 4)-linked D-xylopyranosyl residues, (1 → 2)-linked β-D-xylopyranosyl residues, (1 → 4)-linked β-D-glucopyranosyl residues, (1 → 5)-linked D-arabinofuranosyl residues, (1 → 4)-linked D-xylopyranosyl residues branching at O-2, whereas (1 → 4)-linked D-galactopyranosyl residues branched at O-6. The (1→)-linked β-D-arabinofuranosyl residues play an important role in the formation of branches during structure condensation.

Polysaccharides produced from mushrooms reported for anticancer and immune-modulatory effects on immune system which activates various immune responses that provide protection against numerous unwanted molecules. Ferreira et al. (2015) reviewed aspects of using *Ganoderma* polysaccharide in medical field with appropriate utilization of bioactive molecule. The polysaccharide chains activate immune defense system and accelerate the release of variety of immune-modulatory molecules, i.e., interleukin (IL)-1b, tumor necrosis factor (TNF)-a, IL-6 from human monocyte-macrophages, and interferon (IFN)-c from T lymphocytes. Bioactive compounds, di-2-ethylhexyl phthalate and 1,8-dihydroxy-3-methoxy-6-methyl-anthraquinone, isolated from *Drechslera rostrata* and *Eurotium tonophilum* fungi by a group of scientists (Alasmary et al. 2018). These compounds displayed cytotoxicity against human carcinoma cell lines, colon carcinoma (HCT-116), cervical carcinoma (HeLa), larynx carcinoma (HEp-2), and hepatocellular carcinoma (HepG-2) at the IC_{50} value ranging from 9.5 to 20.3 μg/ml concentration. Li et al. (2017) identified promising strain, *Aspergillus candidus,* for production of a diterpenoid sphaeropsidin A (SphA) biomolecule. The bioactive compound showed antitumor activity against glioblastoma D423 and Gli56 cell lines.

17.2.2 Biocatalysts

Utilization of waste organic material through fungal biomass known as biocatalysis and biotransformation provides useful products with beneficial by-products based on their industrial use. A fungus can demonstrate production of wide variety of enzymes and their enzymatic reaction for valuable product formation.

17.2.2.1 Laccases

Rivera-Hoyos et al. (2013) provided a brief account on fungal laccase enzyme that mediates transformation of aromatic and nonaromatic compound with the release of water molecule and oxygen molecule. Various fungal gene families are available for chemo-enzymatic reaction and helpful in morphogenesis, biodegradation of lignin or dye and pigment biosynthesis and also contribute to sporulation and plant pathogenesis. Laccase structure can be monomer, dimer, or tetrameric glycoprotein based on the presence of copper atoms at their catalytic site which required minimum copper ions for higher activity. These catalytic sites dominantly possess histidine-rich specific amino acid sequence for binding with copper residues. Orlikowska et al. (2018) conducted structural studies of PsLacI and PsLacII isolated from *Pycnoporus sanguineus* CS43 and confirmed variation in novel laccase enzyme structure compared to existing enzyme structures. Due to variation in N-glycosylation site Asn354, enzymatic activity was enhanced with strong substrate binding capacity for further process. Laccase enzyme is an ideal catalyst as it utilizes oxygen with main substrate and produces water molecules as by-product. Laccase enzyme is highly stable at acidic pH and catalyze different enzymatic activities such as dye degradation, decolorization, and pollutant degradation (Kolomytseva et al. 2017).

Wang et al. (2018) isolated a novel extracellular laccase enzyme from white rot fungus *Trametes* sp. F1635 and determined its ability to decolorize toxic dyes, i.e., eriochrome black T (EBT), remazol brilliant blue R (RBBR) and malachite green (MG), in the presence of optimal mediators, violuric acid and acetosyringone, for fasting the reaction. *Streptomyces ipomoeae* CECT 3341 is able to decolorize the dyes, i.e., Acid Black 48, Acid Orange 63, Reactive Black 5, and Azure B dyes through yellow laccase enzyme production in the presence of natural optimal mediators, i.e., 3,5-dimethoxy- 4-hydroxyacetophenone (acetosyringone, AS) and 3,5-dimethoxy-4-hydroxybenzaldehyde (syringaldehyde, SA). The gene laccase *SilA* responsible for laccase production was isolated and modified to possess higher degradation properties under unfavorable condition during the detoxification of dyes (Blanquez et al. 2018). Othman et al. (2018) purified two novel laccases from 3 weeks old fungal culture of *Agaricus bisporus* CU13 using column chromatography. Isoenzyme laccase (Lacc1 and Lacc2) decolorized acid blue dye and Lacc1 was found more thermostable compared to Lacc2 enzyme.

17.2.2.2 Proteases

Protease enzyme plays an important role in agro-industrial field and catalyze the production of pharmaceutical proteins and peptides, cosmetics, and food industry (Sahay et al. 2017; Yadav et al. 2016, 2017, 2018). Protease enzyme helps to digest protein in meat industry and lowers the cost of wastewater treatment. Protease production in fungi, *A. oryzae*, *Penicillium roquefortii*, and *Aspergillus flavipes*, was studied by Novelli et al. (2016). The protease secreted by *A. oryzae* was highly

active compared to the other two microorganisms with strong stability at different temperature (50–90 °C) and pH. *Aspergillus niger* was used for the production of alkaline protease using the substrates, soybean meal (94.7%), and cottonseed meal (106.1%) (Castro et al. 2016).

Salihi et al. (2017) isolated *A. oryzae* CH93 from soil and used for the production of alkaline protease enzyme (47.5 kDa) by utilizing five different substrates, gelatin, azocasein, casein, bovine serum albumin (BSA), and N-acetyl-l-tyrosine ethyl ester monohydrate (ATEE), for maximum enzyme production. *Aspergillus foetidus* was utilized for the production of aspartic protease under suitable acidic condition, pH 5.0 and 50 °C temperature with increased purity of 16.9-fold and 248.1 U g/L specific enzymatic activity (Souza et al. 2017).

Silva et al. (2018) documented the effect of various thermodynamics parameters on the production of alkaline serine protease from *Aspergillus tamarii* URM4634 isolated from soil sample. For pure enzyme, they recorded the thermodynamic parameters (activation energy (E^*d = 49.7 and 28.8 kJ/mol), enthalpy (H^*d = 47.0 and 26.1 kJ/mol), entropy (S^*d = −141.3 and − 203.1 J/mol K), and Gibbs free energy ($92.6 \leq G^*d \leq 96.6$ kJ/mol and $91.8 \leq G^*d \leq 98.0$ kJ/mol). The variation supports the activity rate constant and stability with different temperatures and the protease exhibited higher enzymatic activity.

17.2.2.3 Lignocelluloses

Gupta et al. (2016) and Johansen (2016) explained the production and mechanism of fungal derived lignocellulose enzyme use in biofuel production. Lignocellulose is composed of cellulose, hemicellulose and lignin units and contributes major part of plants. Fungal-derived lignocellulose enzyme consumes lignocellulose biomass as carbon source and produces beneficial by-products. Fungal biomass acts as enzyme repository which degrades lignocellulose, lignin and complex polysaccharides in plants. Paramjeet et al. (2018) reported that biodegradation of lignocellulose is mediated by a group of enzymes that belongs to group I, cellulase-cellobiohydrolase-β-1-4-glucosidase which converts cellulose into glucose; group II, xylanase-β-xylosidase that produce xylose from hemicellulose xylan; and group III, composed of peroxidase and laccase which is able to synthesize oxidized monomers from lignin molecules. Under aerobic and anaerobic condition, *Paenibacillus glucanolyticus* strains SLM1 and 5162 were found to be able to degrade lignocellulose and lignin polymers (Mathews et al. 2016).

17.2.2.4 Chitinases

Fungal-derived chitin and chitosan are important components of agriculture and industrial field such as water treatment, pollution control, genetic engineering due to nontoxicity, and high abundancy. Chitosan is comprised of branched β-(1–4)-linked d-glucosamine and N-acetyl-d-glucosamine, which possess free protonated

amino acid and helps for solubilization in acidic solutions. Fungi also produce chitinase enzyme which hydrolyze the chitosan and ensure the availability of chitin and chitosan for further use. Shehata et al. (2018) studied chitinase and chitosanase enzyme secreted from marine fungus, *Aspergillus griseoaurantiacus* KX010988 and purified using molecular isolation techniques, i.e., ammonium sulfate precipitation, DEAE-cellulose ion-exchange chromatography, and Sephacryl S-300 gel chromatography. They calculated the thermodynamics of the enzyme via activation energy for thermal denaturation ($E_{a,d}$), change of free energy (G_d), enthalpy (H_d), entropy (S_d), and half-life value ($T_{1/2}$) units. Based on the enzymatic activity, *A. griseoaurantiacus* KX010988 can be utilized as biocontrol agent for phytopathogenic fungus, *Fusarium solani*, as it dissolves the fungal chitin membrane and suppress fungal growth in the plants.

Tamreihao et al. (2016) examined the role of chitinase-producing fungus, acidotolerant *Streptomyces* sp. MBRL 10 as plant growth-promoting fungi and biocontrol agent against *Rhizoctonia solani*. Acidotolerant *Streptomyces* sp. was able to produce variety of natural compounds, chitinase, β-1,3-glucanase, lipase, and protease, with phytohormone indole-acetic acid production with release of ammonia in the environment. Bouacem et al. (2018) isolated 59103,12-Da monomer, endo-chitinase enzyme (ChiA-Hh59) from *Hydrogenophilus hirschii* strain KB-DZ44, with high stability in acidic and thermal condition. The catalytic reaction regulated by the ChiA-Hh59 is an endo-splitting catalyst that degrades chitosan for industrial purpose.

17.2.3 Food and Dietary Field

Fungal biomass contributes to the food, dietary, as well as nutraceutical field for derivation of food colorants, polysaccharides, and improved neutraceutical components. Rathore et al. (2017) explained the importance of mushrooms in food industry as the mushroom is a rich source of carbohydrate, proteins and fats with higher nutritional values with unsaturated fatty acid upon linoleic acid, oleic acid and palmitic acid (saturated fatty acids). Mushrooms are reported for their highly nutritional content of antioxidants, β-glucans and triterpenoids. Further, it has been reported that fungal biomass produces variety of bioactive substance that acts as food ingredients or supplements such as fungal pigments, polyunsaturated fatty acids, flavor enhancers and vitamins with acidic/alkali food regulators (Dufosse 2018). The report says that the fungi, i.e., *Monascus purpureus*, *Talaromyces albobiverticilliu*, *Talaromyces atroroseus*, *Ashbya gossypii*, *Penicillium purpurogenum*, *Penicillium oxalicum* and *Blakeslea trisporas*, are able to produce the yellow monascin and ankaflavin, the orange monascorubrin, rubropunctatin, the red monascorubramine and rubropunctamine, carotenoid lycopene, riboflavin and azaphilone pigments. These pigments can be utilized as food colorants which can replace toxic colorants. Tropical marine fungi, *T. albobiverticillius*, isolated from Reunion Island, Indian Ocean, was used for red pigment production (Venkatachalam et al. 2018b).

Venkatachalam et al. (2018a) analyzed *T. albobiverticillius* 30,548 from red coral and identified the presence of three bioactive compounds, N-threonine-

monascorubramine, N-glutaryl-rubropunctamine,and PP-O, and first time reported as that NGABA-PP-V (6-[(Z)-2-Carboxyvinyl]-N-GABA-PP-V) molecule responsible for red color in coral. Wu et al. (2018) reported *A. niger* and *Rhizopus oryzae* for enhancement of β-glucan extraction from oat bran and increased the availability of nutritional element.

17.2.4 Agriculture Aspect

Fungal diversity occupies a major part of the soil ecosystem where it has some beneficial impact on food crops and plants, for example, boosting plant defense system and phytohormone production as plant growth-promoting microorganisms and biocontrol agents.

17.2.4.1 Plant Growth-Promoting Fungi

Fungi provide nutritional elements from soil and in exchange can reside on host plant in symbiotic relationship. Small and Degenhardt (2018) described five major classes of plant growth regulators, gibberellic acid, cytokinin, auxin, ethylene, and abscisic acid, which are phytohormones and regulate plant growth with different mechanisms. Cytokinin, auxin and gibberellic acid are important for plant development and growth, whereas ethylene is an essential element for fruit ripening and abscisic acid is responsible for stimulation of plant defense system to fight against pathogenic microbiota. Vergara et al. (2018) studied colonization of four dark septate fungi *Pleosporales* on rice plants that helps in rice plant growth by availing macronutrients and increased the nutrient uptake by restoring nitrate contents in the root soil. The entomopathogenic fungi *Beauveria bassiana*, *Metarhizium anisopliae*, and *Metarhizium robertsii* were utilized to analyze the plant growth-promoting effects on growth and yield of the soybean *Glycine max* (L.) Merr (Russo et al. 2018).

Endophytic fungus, *Thermomyces* sp., isolated from hot desert-adapted delile (*Cullen plicata*) roots that displayed beneficial effects on growth of cucumber plants by accelerating production of total sugars, flavonoids, saponins, soluble proteins and antioxidant enzymes (Ali et al. 2018). Kumar et al. (2018) analyzed effects of entomopathogenic fungus, *Lecanicillium psalliotae*, on *Elettaria cardamomum* plant growth traits. Fungus, *L. psalliotae*, promoted plant growth by producing auxin, solubilizing phosphate, zinc and increased nutrient bioavailability.

17.2.4.2 Biocontrol

Due to excessive use of chemical fertilizer, phytopathogens have become resistant to the fertilizer and the mutated microbial strains, can be problem for further crop production. Crop protection is an effective issue which requires use of beneficial

microbes as biofertilizer. Biofertilizers have a positive impact on soil microbiome and can be used as plant growth-promoting microbes or increasing phytohoromone production and boost plant host defense system. Ghorbanpour et al. (2018) reported the main strategies behind biocontrol mechanism, i.e., induce antibiosis, mycoparasitism, microbial competition, mycovirus-mediated cross protection (MMCP) and induced systemic resistance (ISR) to control pathogenic microbiota.

Entomo-pathogenic fungi provide plant protection against plant disease and pathogenic microbes by increasing plant growth and development and rhizosphere colonization. The decay fungus, *Chondrostereum purpureum*, was used as biocontrol agent as it secretes various enzymes and penetrates trees root and causes white rot disease in broad-leaved trees (Hamberg et al. 2018). Nematophagous fungi, *Pochonia chlamydosporia* and *Duddingtonia flagrans*, were used as biocontrol agents for nematode infection with plant growth-promoting traits such as increased nutrient uptake and phosphate solubilization (Monteiro et al. 2018). A non-aflatoxigenic *A. flavus* ARG5/30 was isolated from maize field and used as biocontrol agent against *Aspergillus* sp. through competition strategy at maize agroecosystem in Argentina (Zanon et al. 2018).

17.2.5 Environmental Issues

Fungi have many beneficial effects on the environment, especially for biodegradation, bioconversion and bioremediation, and reduce greenhouse gas emission. Researchers have proposed various methodologies to compete with pathogenic microbes and reduce production of harmful products from different industries.

17.2.5.1 Mycoremediation

Industrial effluents generated from the industries are harmful to the environment due to the presence of highly toxic metals, metalloids, radio nucleotides, organic metals or pharmaceutical matter. These elements have deleterious effects on the environment that depends on the physiochemical properties and the metal group, which influence the microbial consortia in the soil and aquatic ecosystem. Sahmoune (2018) reviewed the impact of fungus, *Streptomyces rimosus*, on biosorption of heavy metals, i.e., lead and iron. The biosorption process is comprised of two main steps, adsorption and desorption. *S. rimosus*-mediated lead (Pb^{2+}) and iron (Fe) metal biosorption from pharmaceutical effluents is influenced by various environmental factors such as temperature, pH of the solution, metal ion concentration, and fungal biomass. The FTIR results concluded that the presence of carboxyl group in fungal biomass boosted the biosorption process.

Long et al. (2018) performed removal of nickel ions (Ni^{2+}) from industrial effluent using dead *Streptomyces roseorubens* SY fungal biomass. Langmuir isotherm was used to determine the maximum capacity of fungal biomass to absorb metal from the solution and found to be 208.39 mg/g at 313 K, respectively. Uranium ($[UO2]^{2+}$) exists in different forms to stabilize in the environment, free uranium (UO_2^{2+}), uranyl carbonate or uranyl phosphate (bind with inorganic ligand) and uranyl fulvate. Availability of uranium ion can be channelized by biosorption of metal ion on fungal biomass mediated through fungal metabolites. Release of metal complex from the fungal biomass depends on the ligand binding efficacy and coordination chemistry (Ogar et al. 2014).

Lee et al. (2014) explained the biotechnological method for removal of polycyclic hydrocarbon (PAH) compounds (phenanthrene, anthracene, fluoranthene, pyrene) generated as xenobiotics through mining process. The process of pollutant removal is categorized based on the type of pollutants and the release of lignocellulytic enzymes, gallic acid reaction and decolourization for the dye. *Coriolopsis byrsina* strain APC5 was reported for removal of PAH by Agrawal and Shahi (2017). They studied the secretion of ligninolytic enzyme for degradation of pyrene with 96.1% and identified the chemical groups responsible for degradation using FTIR.

Vieira et al. (2018) optimized the fungal biodegradation of PAH (pyrene and benzo[a]pyrene) degradation using marine-derived basidiomycetes. The basidiomycete, *Marasmiellus* sp. CBMAI 1062 was highly active during biodegradation and FTIR analysis showed the enzymatic involvement of the cytochrome P450 system and epoxide hydrolases. Dye removal from the solution was mediated via fungal biomass, *Pleurotus ostreatus* (BWPH), *Gloeophyllum odoratum* (DCa) and *Polyporus picipes* (RWP17) for Brilliant green and Evans blue dyes. Fungus, *P. picipes*, showed the highest immobilization rate with decreased phytotoxicity and less toxic xenobiotic by-product (Przystas et al. 2018). Wang et al. (2017b) isolated white rot fungus, *Ceriporia lacerata* from decayed mulberry branches that was able to decolorize and degrade Congo red dye under favorable conditions. Report says that abundancy of manganese peroxidase (MnP) enzyme is responsible for biotransformation of Congo red dye into naphthylamine and benzidine product.

17.2.5.2 Greenhouse Gas Emission

Soil is a rich source of microbial consortia, and arbuscular mycorrhizal fungi are prominent due to their symbiotic relationship with the host plants. AMF alters the soil nutrient availability by providing high surface area for nutrient adsorption, maintains acidic and alkali condition, changes water level by providing water tolerance and monitors the drainage level for the contaminated water into groundwater. The reduction of greenhouse gas emission is one of the main problems solved by AMF through the assimilation of nitrogen uptake, carbon sequestration and changes soil moisture which affect mineralization, nitrification and denitrification processes. The release of nitrous oxide (N_2O) during nitrogen cycle in the soil can be

minimized by applying AMF on the field. AMF provides carbon to the soil and reduces N_2O production. AMF *Rhizophagus irregularis* applied to maize field maximized the nitrogen uptake and reduced greenhouse gas emission (Storer et al. 2017). Wang et al. (2016) set up a 2-year field experiment in Shendong coal mining subsidence area, China, and four crop plants (wild cherry (*Prunus discadenia Koebne* L.), *Cerasus humilis* (*Prunus dictyoneura Diels* L.), shiny leaf yellow horn (*Xanthoceras sorbifolium Bunge* L.) and apricot (*Armeniaca sibirica* L.)) were planted in the agriculture land. They reported significant increase in carbon sequestration and temperature sensitivity after treatment. AMF supports plant growth by increasing leaf size and chlorophyll content for photosynthesis process.

17.2.6 Biotechnology

Fungi can be used as prominent biotechnological tool for the production of economically important biomolecule at large scale to minimize the burden on nonrenewable sources (Fig. 17.1).

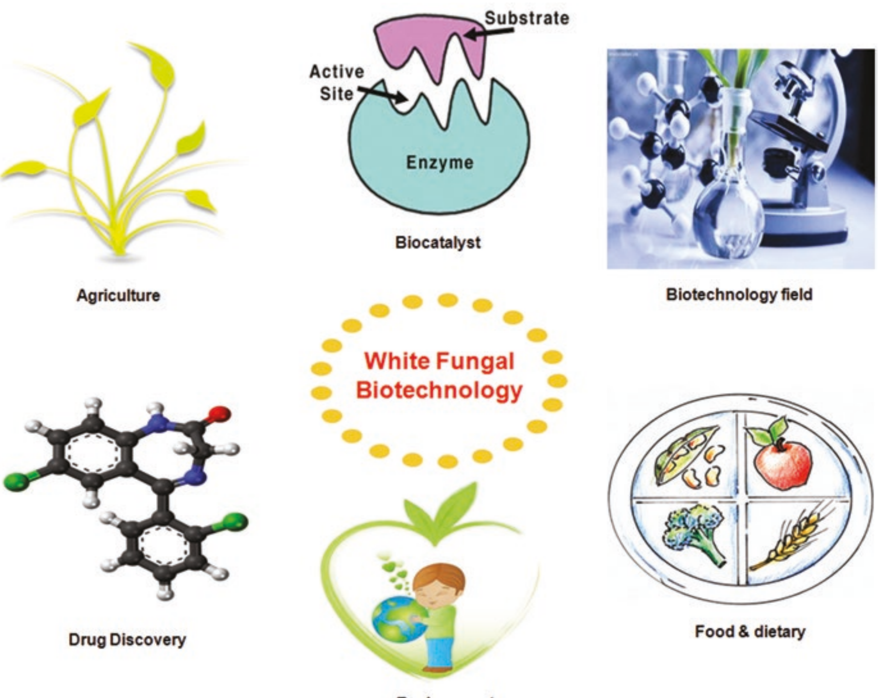

Fig. 17.1 Brief illustration of white fungal biotechnology on human health and science society

17.2.6.1 Recombinant Protein Production

Filamentous fungi, *Penicillium*, *Trichoderma*, *Aspergillus*, *Fusarium*, *Rhizopus* and *Mucor* species are chemotrophic fungi that rely on organic carbon and energy sources for food. These fungi are able to produce variety of bioactive compounds including pigments, enzymes, polysaccharides, and lead compounds for medicinal and industrial use. These fungi are also involved in biological process, biotransformation, biodegradation and nutrient recycling in the soil (Ward 2012). Filamentous fungi can be used as a good recombinant protein-producing microorganism but are less preferable due to their intra- and extracellular enzyme production which may degrade the newly formed proteins.

Ward (2012) explained problem with recombinant protein production in fungi and how to minimize with minimal impact on fungal cell with some genetic modifications in fungal genome. *Aspergillus nidulans* A773 was used for co-production of two enzymes, xylanase and arabinofuranosidase using soybean fiber and utilized the same substrate for the production of xylooligosaccharides. The composition of produced xylooligosaccharides was 138.36 mg/g xylobiose, 96.96 mg/g xylotriose, and 53.04 mg/g xylotetraose which were obtained after enzymatic reaction of 9 h (Pereira et al. 2018). Chesini et al. (2018) enhanced the production of recombinant acid stable exoinulinase from *Aspergillus kawachii* and genetically engineered enzyme was cloned in *Pichia pastoris* system. Itulin substrate was used to determine the enzymatic activity of overproduced recombinant protein that possesses fructosyltransferase activity and suggests carbohydrate processing. The Hg^{2+} ions completely inhibit the enzymatic activity but the protein is highly stable at 55 °C for 3 h.

Madhavan et al. (2017) performed experiment to determine the overexpression of heterogeneous protein in *Aspergillus unguis* NII 08123, a filamentous fungi using variety of promoters and selective markers. A complex was formed for protein expression, comprised of glyceraldehyde 3 phosphate dehydrogenase promoter (Pgapd) and tryptophan synthase transcription terminator (TtrpC). Hygromycin resistance gene (hph) was used as selection marker and green fluorescent protein (GFP) gene from *Aequorea victoria* placed as standard marker protein. Recombinant therapeutic protein-human interferon beta (HuIFNb) overexpressed in *A. unguis* is a successful example of recombinant protein production in fungal system.

17.2.6.2 Biofuel Cell Factories

Biofuel production can be maximized by using fungi for fermentation of plant polysaccharides due to high enzyme content with high thermostability at high processing temperature and enzyme inhibitor resistance. Makhuvele et al. (2017) isolated 101 fungi which were further utilized for ethanol production using thatch grass as substrate. Among the isolated fungi, *Aspergillus* species was reported for higher enzyme secretion during the process such as endo-glucanase, xylanase and mannanase. Degradation of soda lignin from cedar using various white rot and leaf-litter

fungi was monitored under experimental conditions. The isolated white rot fungi are *A. gypsea*, *Fomes fomentarius* (L.) Fr., *Ganoderma austral* (Fr.) Pat., *Ganoderma neojaponicum Imazeki*, *Heterobasidion ecrustosum Tokuda* T. Hatt. and Y.C. Dai, *Hymenochaete yasudai*, *Hyphodontia breviseta* (P. Karst.) J. Erikss., *Phanerochaete sanguinea* (Fr.) Pouz., *Phanerochaete velutina* (DC.) Parmasto, *Phlebia tremellosa* and *S. furfurella* with 3 leaf litter fungi, *Agrocybe praecox* (Pers.) Fayod, *Marasmius cryptomeriae*, and *Stropharia rugosoannulata* Farl. ex Murrill. Based on NMR results, they concluded degradation of aromatic polymer (high persistent coniferous lignin) was performed via different mechanisms involved for conversion of lignin into usable biofuel (Saito et al. 2018).

17.2.6.3 Secretomics

Mendoza et al. (2018) reviewed the role of secretome in *Trichoderma* species with respect to their symbiotic relationship with host plants. *Trichoderma* possess secretome that releases diversified enzyme in the environment which help it to attach with plant root, avail nutrient from the soil and antagonistic activity against plant pathogen. Bioactive molecule such as hydrophobin and swollenin allows attachment of the species to the plant root and minimizes plant defense system to initiate plant root colonization. Ji et al. (2012) investigated *Trametes trogii* MT for lignocellulose-degrading properties and analyzed secretome system for the presence of beneficial enzymes. The secretome analysis showed the presence of laccase and manganese peroxidase enzyme in high abundance which degrades lignin, cellulose as well as hemicellulose. Berrin et al. (2017) studied fungal secretomics to analyze the biological functions of lytic polysaccharide monooxygenases highly secreted by white rot fungi, *Pycnoporus coccineus*, *Phanerochaete chrysosporium*, and *Ceriporiopsis subvermispora*. Cai et al. (2017) conducted a comparative study on the production of enzymatic system in *Lentinula edodes* on different substrates, micro crystallized cellulose, lignosulfonate and glucose. The secretome analysis displayed a wide range of enzymes (lipase, protease, peptidase, CAZyme and oxidoreductase) production from fungi in liquid medium. The cellulose-containing medium revealed the presence of polysaccharide hydrolytic enzymes, endo-β-1,4-glucanase, α-galactosidase, polygalacturonase, and glucoamylase, whereas lignocellulolytic enzymes were present in glucose culture compared to lignosulfonate and cellulose medium (Table 17.1).

17.3 Genetic Engineering of Metabolic Pathways

Fungi are rich source of secondary metabolites and produce structurally different chemical molecules that are beneficial for medical and industrial fields (organic matter fermentation, protein production, and secondary metabolism). Primary and secondary metabolite production is mediated via metabolic pathways regulated by

Table 17.1 Beneficial applications of fungi in the white fungal biotechnology field

Beneficial application	Important fungi	References
Drug discovery		
Antimicrobials	*Aspergillus versicolor*, *Colletotrichum gloeosporioides*, *Cryptococcus neoformans*, *Curvularia* sp., *Diaporthe* sp., *Eupenicillium levitum* CJ-MR2, *Fusarium* sp. PSU-ES73, *Hypocreales* sp. PSU-ES26, *Penicillium* sp. PSU-ES43, *Simplicillium lamellicola*, *Stephanonectria* sp. PSU-ES172, *Talaromyces marneffei*, *Trichoderma* spp. PSU-ES8	Dang et al. (2014), Eze et al. (2018), Nurunnabi et al. (2018), Ribeiro et al. (2018), Richardson et al. (2015), Supaphon et al. (2013), Supaphon et al. (2018) and Wang et al. (2017a)
Antioxidant/ anticancer/antitumor	*Aspergillus candidus, Drechslera rostrata*, *Eurotium tonophilum*, *Ganoderma*, *Phellinus linteus*, *Phomopsis* sp.	Alasmary et al. (2018), Ferreira et al. (2015), Li et al. (2017), Minarni et al. (2017) and Pei et al. (2015)
Biocatalysts		
Laccases	*Agaricus bisporus*, *Pycnoporus sanguineus*, *Streptomyces ipomoeae*, *Trametes*	Blanquez et al. (2018), Orlikowska et al. (2018), Othman et al. (2018), and Wang et al. (2018)
Proteases	*Aspergillus flavipes*, *Aspergillus foetidus*, *Aspergillus niger*, *Aspergillus oryzae*, *Aspergillus tamari*, *Penicillium roqueforti*	Castro et al. (2016), Novelli et al. (2016), Salihi et al. (2017), Silva et al. (2018), and Souza et al. (2017)
Lignocelluloses	*Paenibacillus glucanolyticus*	Mathews et al. (2016) and Paramjeet et al. (2018)
Chitinases	*Aspergillus griseoaurantiacus*, *Hydrogenophilus hirschii*, *Streptomyces* sp.	Bouacem et al. (2018), Shehata et al. (2018) and Tamreihao et al. (2016)
Food and dietary		
	Ashbya gossypii, *Blakeslea trispora*, *Monascus purpureus*, *Penicillium oxalicum*, *Penicillium purpurogenum*, *Talaromyces atroroseus*, *Talaromyces albobiverticillius*	Dufosse (2018)
	A. niger, *Rhizopus oryzae*, *T. albobiverticillius*	Venkatachalam et al. (2018a, b) and Wu et al. (2018)
Agricultural aspect		
Plant growth promotion	*Beauveria bassiana*, *Lecanicillium psalliotae*, *Metarhizium anisopliae*, *Metarhizium robertsii, Pleosporales*, *Thermomyces* sp.	Ali et al. (2018), Kumar et al. (2018), Russo et al. (2018) and Vergara et al. (2018)
Biocontrol	*A. flavus*, *Chondrostereum purpureum*, *Duddingtonia flagrans*, *Pochonia chlamydosporia*	Hamberg et al. (2018), Monteiro et al. (2018) and Zanon et al. (2018)

(continued)

Table 17.1 (continued)

Beneficial application	Important fungi	References
Environments		
Mycoremediation	*Ceriporia lacerata*, *Coriolopsisbyrsina*, *Marasmiellus* sp. *Pleurotus ostreatus*, *Gloeophyllum odoratum*, *Polyporus picipes*, *Streptomyces rimosus*, *Streptomyces roseorubens*	Agrawal and Shahi (2017), Long et al. (2018), Przystas et al. (2018), Sahmoune (2018), Vieira et al. (2018) and Wang et al. (2017b)
Greenhouse gas emission	*Rhizophagus irregularis*	Storer et al. (2017) and Wang et al. (2016)
Biotechnology		
Recombinant protein production	*Aspergillus unguis*, *Aspergillus kawachii*, *Aspergillus nidulans*, *Pichia pastoris*	Chesini et al. (2018), Madhavan et al. (2017) and Pereira et al. (2018)
Biofuel cell factories	*A. gypsea*, *Agrocybe praecox*, *Aspergillus* species, *Fomes fomentarius*, *Ganoderma austral*, *Ganoderma neojaponicum*, *Heterobasidion crustosum*, *Hymenochaete yasudai*, *Hyphodontia breviseta*, *Marasmius cryptomeriae*, *Phanerochaete sanguinea*, *Phanerochaete velutina*, *Phlebia tremellosa*, *Stropharia rugosoannulata*	Makhuvele et al. (2017) and Saito et al. (2018)
Secretomics	*Ceriporiopsis subvermispora, Lentinula edodes*, *Phanerochaete chrysosporium*, *Pycnoporus coccineus*, *Trametestrogii*, *Trichoderma*	Berrin et al. (2017), Cai et al. (2017), Ji et al. (2012) and Mendoza et al. (2018)

gene cluster associated with chromosomal region. Metabolic pathway is regulated via variety of enzymatic reactions which further produce bioactive compound and by-product (Wakai et al. 2017). Genetic engineering is a promising approach to alter fungal gene expression and produce high level of expected protein molecule. Heterologous protein expression, bioconversion and chemical modification of metabolic pathway component may enhance the expression of particular gene.

Bomke and Tudzynski (2009) reviewed gibberellin production in fungi via biosynthetic metabolic pathway. Biosynthetic pathway assigned for gibberellic acid production was first reported in *Gibberella fujikuroi* and therefore plays an important role in plant growth. Further *Phaeosphaeria* spp. and *Sphaceloma manihoticola* were also reported for the presence of GA biosynthetic pathway. Activation of gibberellic acid synthesis is initiated by condensation of precursor molecule, acetyl-CoA, with isopentenyl diphosphate (IPP) molecule and produces farnesyl diphosphate (FDP). Two geranylgeranyl diphosphate synthases GGS1 and GGS2 activate the production of geranylgeranyl diphosphate (GGDP) molecule. GGDP is highly important molecules which modulate production of specific intermediates,

ent-kaurene by ent-copalyl diphosphate (CPP) and transform into ent-kaurenoic acid (KA) via cyclization and oxidation reaction, respectively. Intermediate ent-kaurenoic acid now undergoes hydroxylation process at 7β-position and produces ent-7-α-hydrokaurenoic acid to construct a ring B. GA_{12} converts into GA_{14} through hydroxylation at 3-β-position and further oxidized to produce GA_{14}. The gene clusters responsible for GA biosynthetic pathway modulation are geranylgeranyl diphosphate synthase (*ggs*1 and *ggs*2) genes.

Saiardi et al. (2018) studied the importance of inositol phosphate metabolic pathway in fungi *Cryptococcus neoformans* and *Trypanosoma brucei* for fungal survival and virulence. The report says the deletion of specific metabolic pathway is highly recommendable for reduction of fungal pathogenicity in the host. The common structure for IPMK is made up of an N-terminal domain, a larger C-terminal domain, and a small inositol-binding domain with two alpha-helices. Inositol phosphate metabolic pathway involves two important secondary messengers known as diacylglycerol (DAG) and the calcium release factor I(1,4,5)P3. Production of inositol tetrakisphosphate I(1,3,4,5)P4 was regulated through phosphorylation of I(1,4,5)P3 by IP3–3 kinase. Dephosphorylation of IP4 produces IP3 I(1,3,4)P3 molecule mediated by inositol polyphosphate-5-phosphatase (5-Ptase) enzyme. An intrinsic-1-phosphatase activity produces I(3,4,5,6)P4 which acts on calcium-activated chloride channels. Hidayat and Yanto (2018) examined tropical fungus *Trametes hirsuta* D7 for its ability to degrade polycyclic aromatic hydrocarbons (phenanthrene, chrysene and benzopyrene (BaP)) on solid media. They conducted the degradation assay mediated by the strain grown with monooxygenase inhibitor, piperonyl (an inhibitor of P450 monooxygenase), and found the presence of five different metabolites during the process. Based on synthesized product during degradation, authors predicted involved chemical reaction and presence of novel metabolic pathway for degradation reaction. The chemical reaction starts with the oxidation process of phenanthrene to phenanthrene 9,10-dihydrodiol (I) which further oxidized with 9,10-phenanthrene quinone to form 2,2′-diphenic acid (II). Conversion of 2,2′-diphenic acid to form benzoic acid (III) and 2,2′-hydroxybenzoic acid (IV) lead to the formation of 1,2-benzenedicarboxylic acid (V). Genetic engineering of metabolic pathways provides easy construction of DNA segments, availability of circular plasmids, fasten the transformation and integration efficiency for further cassette involved in the production of diversified bioactive molecules. Sarkari et al. (2017) conducted a study, genetic engineering, for gene integration in genome of *A. niger* for production of heterogeneous protein, aconitic acid. They integrated the shortened AMA1 peptide (metabolic pathway variants) using Golden Gate cloning and CRISPR/Cas9 strategy to maximize the DNA construction and incorporation of plasmid into fungal genome (Fig. 17.2).

Pullulan is a simple homopolysaccharide molecule with advantages of low toxicity, higher viscosity and digestibility. *Aureobasidium pullulans* CGMCC1234 produces pullulan molecule with a cascade of chain reaction which requires involvement of central metabolic pathways, Embden-Meyerhof pathway (EMP) and pentose phosphate pathway (PPP). PPP pathway require enzymatic activity which is catalyzed

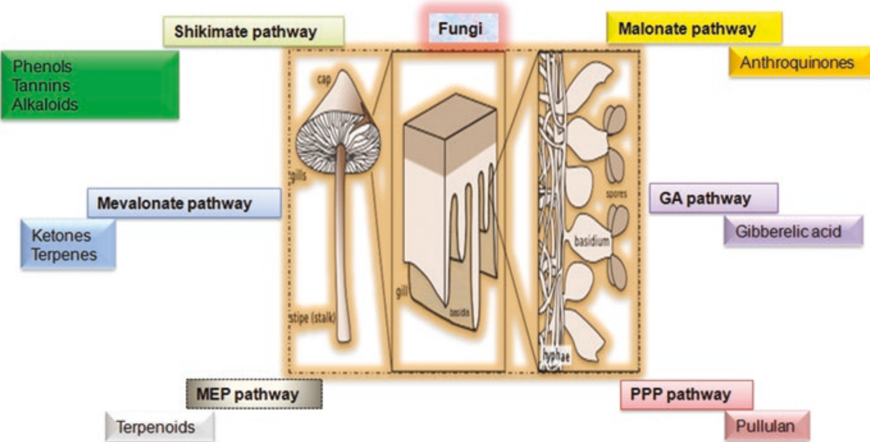

Fig. 17.2 Graphical representation of metabolic pathways in fungi for biosynthesis of bioactive molecules

by glyceraldehyde-3-phosphate dehydrogenase (GAPDH) for ATP generation. Iodoacetic acid is an inhibitor of pullulan production and may reduce fungal cell growth. Sheng et al. (2015) concluded that iodoacetic acid interrupts pullulan production and make feasibility of providing carbon to the PPP pathway as it involves oxidation of glyceraldehyde 3-phosphate to glyceraldehyde-1,2-diphosphate.

17.4 Conclusion and Future Perspective

White fungal biotechnology is composed of wide variety of fields such as agriculture, medical, food and dietary, biocatalysis, environment and biotechnology. The fungi *Aspergillus*, *Trichoderma*, *Penicillium*, and *Pleurotus* were highly important fungal group which can be utilize for the production of different antibiotics, enzymes and peptides useful in medical and industrial field. Secretomic analysis is one of the prominent hub to identify secretion of enzymes and the production can be maximized by using genetic engineering approachs. Understanding of fungal genome is required to know the biology of silent secondary metabolites genes which involves complex processes like marker recycling and genome editing may enhance the bioproduction of beneficial molecules. Alteration of any biological component in the metabolic pathways increase the production of variety of bioactive compounds and metabolic enzymes.

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