Fungal Biology

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Recent Advancement in White Biotechnology Through Fungi

Volume 2: Perspective for Value-Added Products and Environments

Springer

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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Foreword

White biotechnology is industrial biotechnology dealing with various biotech products through applications of microbes. The main application of white biotechnology is commercial production of various useful organic substances, such as acetic acid, citric acid, acetone, glycerine, etc., and antibiotics, like penicillin, streptomycin, mitomycin, etc., and value-added product through the use of microorganisms especially fungi and bacteria. The value-added products included bioactive compounds, secondary metabolites, pigments, and industrially important enzymes for potential applications in agriculture, pharmaceuticals, medicine, and allied sectors for human welfare. In the twenty-first century, humans acquired skills 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. 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 pharmaceuticals (medical/red biotechnology). In contrast, the production of bioactive compounds through fermentation or enzymatic conversion is known as industrial or white biotechnology. The beneficial fungal strains may play important role in agriculture, industry, and medical sectors. The beneficial fungi play a significant 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. The discovery of antibiotics is a milestone in the development of white biotechnology. 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. The fungal strains and bioactive compounds also play important role in the environmental cleaning.

The present book volume on *"Recent Advancement in White Biotechnology Through Fungi" Volume 2: Perspective for Value-Added Products and Environments* is a very timely publication, which provides state-of-the-art information in the area of white biotechnology, broadly involving fungal-based value-added products and applications of fungal communities for sustainable environments. The book volume comprises 16 chapters. Chapter 1 by Kour et al. describes agriculturally and industrially important fungi and their potential biotechnological applications. Chapter 2 presented by Dailin et al. highlights fungal phytases and their biotechnological applications in food and feed industries. Chapter 3 by Banik et al. describes about probiotic fungal strains, their mechanism of actions, health beneficial effects, and also their efficacy in the treatment of various diarrheal, skin, and vaginal complications. Chapter 4 by Challa et al. highlights the opportunities and challenges of fungal white biotechnology to meet food security. Roy and Banerjee describe the production of hydrocarbon and hydrocarbon-like compounds along with other quality volatile organics with high potential to be used as both "green chemicals" and fuels from endophytic fungi in Chap. 5. Chapter 6, by Parasuraman and Siddhardha, gives details of functional genomics and proteomics for the isolation and production of novel natural value-added metabolites from fungal community. Chapter 7 authored by Gholami-Shabani et al. highlights current knowledge about fungal natural products including primary and secondary metabolites, their biosynthetic pathways, and brief examples of each class of compounds including their bioactivities. In Chap. 8, Singh and colleagues describe in detail about the variety of secondary metabolite produced, its synthesis strategies via chemical and heterologous mode, as well as their biological applications. Enespa et al. highlight the fungal production of novel secondary metabolites with antimicrobial activity against plant and human pathogenic fungal and bacterial strains in Chap. 9. Kumar et al. explain about pigments produced by soil fungi and their potential applications in medical, textile coloring, food coloring, and cosmetics in Chap. 10. The endophytes that may contribute to their host plant and for the pharmaceutically important bioactive substances, as the search for better chemotherapeutic agents remains an important challenge, have been described by Carvalho et al. in Chap. 11. Chapter 12 by Sharma and Salwan describes the extracellular enzymes from *Trichoderma* and their role in the production of biofuel from nonedible biomass. Raven et al. highlight the third-generation biofuels generated with the assistance of fungi in Chap. 13. Diwan and Gupta discuss the advent of single cell oil and realization of its multiple prospects and possibilities in Chap. 14. Recent advancement and the way forward for *Cordyceps* have been discussed in Chap. 15 by Chaubey et al. Finally, Rashmi et al. describe about the status of synthetic biology for production of value-added products and bioactive compounds from fungi for advancements of fungal white biotechnology in Chap. 16.

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 the students, researchers, scientists, and academicians. 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 Ph.D. 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 until to date. He had worked as Professor of Biotechnology at Indian Institute of Technology (IIT), Roorkee (2003–2011); Founder Director of Biotechnology Centre, Punjab Agricultural University (PAU), Ludhiana (1992–2003); Senior Scientist and Wheat Breeder-cum-Director at PAU's Regional Research Station, Gurdaspur (1979–1990); Research Fellow at the Friedrich Miescher Institute (FMI), Basel, Switzerland (1976–1979); and D. F. Jones Postdoctoral Fellow at the University of California, Riverside, USA (1975–1976). Dr. Dhaliwal was elected as Fellow at National Academy of Agricultural Sciences, India (1992), and has worked as Visiting Professor in the Department of Plant Pathology, Kansas State University, Kansas, USA, (1989) and Senior Research Fellow at the International Maize and Wheat Improvement Center (CIMMYT), Mexico (1987). He has many national and international awards on 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 and conferences and abstracted, 18 popular articles, and 2 books/bulletins/manuals. His important research contributions are identification of new species of wild diploid wheat *Triticum urartu* and gathered evidences to implicate *T. urartu* as one of the parents of polyploid wheat; Team Leader in the development of seven wheat varieties, namely, 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-resistant wheat cultivar. Dr. Dhaliwal had the membership of several task forces and committees of the Department of Biotechnology, Ministry of Science and Technology, Government of India, New Delhi, and is Chairman of Project Monitoring Committee for Wheat Quality Breeding, Department of Biotechnology, Ministry of Science & Technology, Government of India (2007–2010); Chairman of the Project Monitoring Committee in "Agri-biotechnology" of the Department of Biotechnology, Government 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 onward).

Preface

White biotechnology is drawing much attention as a solution to produce value-added product for human welfare. Fungi are used to synthesize functional bioactive compounds, hydrolytic enzymes, and compounds for plant growth promotion, biocontrol, and other processes for agriculture, medicine, industry, pharmaceuticals, and allied sectors. White fungal biotechnology is an emerging field in science arena that supports revealing of novel and vital biotechnological components. 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, pigments, and peptides useful in medical and industrial fields.

The present book on "*Recent Advancement in White Biotechnology Through Fungi" Volume 2: Perspective for Value-Added Products and Environments* covers agriculturally and industrially important fungi producing value-added products for agriculture, industry, and environments. The fungal community from different habitats such as from extreme habitats as well as plant associated having capability to produced extracellular enzymes, secondary metabolites and bio-active compounds are useful for diverse processes targeted at therapeutics, diagnostics, bioremediation, agriculture, industries and environments. This book volume will be immensely useful to biological sciences, especially to microbiologists, microbial biotechnologists, biochemists, researchers, and scientists of fungal biotechnology. We are honored that the leading scientists who have extensive, in-depth experience and expertise in fungal system 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 agricultural industrial and environmental applications of fungi.

We are grateful to the many people who helped to bring this book to light. Editors wish to thank Dr. Eric Stannard, senior editor, Botany, Springer; Dr. Vijai Kumar Gupta and Maria G. Tuohy, series editors, Fungal Biology, Springer; and Mr. Rahul Sharma, project coordinator, Springer, for generous assistance, constant support, and patience in initializing the volume. Dr. Ajar Nath Yadav gives special thanks to

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Young Scientist Award, two Poster Presenter Awards, Fellowship Award awarded by International Consortium of Contemporary Biologist, and Dr. V. P. Agarwal Gold Medal. Dr. Arti has lifetime membership of Indian Science Congress Association, Biotech Research Society of India, Zoological Society of India, and International Consortium of Contemporary Biologist.

Chapter 1
Agriculturally and Industrially Important Fungi: Current Developments and Potential Biotechnological Applications

Divjot Kour, Kusam Lata Rana, Neelam Yadav, Ajar Nath Yadav, Joginder Singh, Ali A. Rastegari, and Anil Kumar Saxena

1.1 Introduction

Fungi are chemoheterotrophic organisms and are known to be present in subaerial and subsoil environments. They are known to play a major role as decomposers, simultaneously being important animal and plant mutualistic symbionts as well as pathogens, further being the spoilage organisms of natural as well as manufactured materials (Burford et al. 2003; Gadd 1999, 2006, 2007). They also play a chief role in maintaining soil structure, due to their filamentous branching growth and frequent production of the exopolymer. Most of the fungi possess a filamentous growth habit and some are polymorphic, occurring as both filamentous mycelium and unicellular yeasts or yeast-like cells (Gadd 2007; Gorbushina et al. 2002, 2003). The filamentous mode of growth provides them the capability to adapt to both exploitative or explorative growth strategies, and the formation of linear organs of

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aggregated hyphae for protected fungal translocation (Fomina et al. 2005; Gadd 2007). Interestingly, the earliest fossil of filamentous fungal remains appear to be from the mid- to late Precambrian (Gadd 2007) and have been revealed to be extremely diverse by Devonian, times, when forms belonging to major groups and even some genera present today are found (Gadd 2007; Heckman et al. 2001). Since that time fungi have been known to be the components of the microbial communities of any terrestrial environment (Hawksworth 2001) which may either be hostile habitats such as the Arctic and Antarctic and hot deserts or may be metal-rich and hypersaline soils (Burford et al. 2003). The majority of fungi have been demonstrated to inhabit soil environments, which are known to be apparently much more hospitable as compared to the bare rock surfaces. Fungal communities in soil are diverse, where they may occur as plant and animal pathogens, free-living or in symbiotic associations, as well as unicellular yeasts (Gadd 2007). They may be present inside the plants which are known as the endophytic fungi where they may reside without causing any harmful effect to the host plant. Plants are in fact considered to be a major reservoir of abundance of endophytes (Rana et al. 2016a, b, 2017). It has been estimated that more than one million fungal species inhabit different genera of the plant which reflects on hyperdiversity of endophytic fungi (Bilal et al. 2018; Strobel and Daisy 2003). Endophytic fungi are known to be one of the best sources of natural bioactive compounds which have potential applications in diverse fields such as food industry, medicine, and agriculture (Bilal et al. 2018; Strobel et al. 2004; Verma et al. 2009; Yadav et al. 2015b).

Numerous endophytes have been examined for their capability to produce metabolites which promote the growth of the plants (Verma et al. 2013, 2014). Further, there is rhizospheric fungi which also play an important role in plant growth promotion by different mechanisms such as production of diverse plant growth regulators; making availability of various nutrients to the plants such as phosphorus, potassium, zinc etc.; and production of the siderophores and diverse hydrolytic enzymes; furthermore, these also help the plants to overcome abiotic stress conditions such as salinity, drought, and high or low temperature, and all these characteristics make them good source to be used as biofertilizers (Saxena et al. 2015a; Suman et al. 2016; Verma et al. 2016b; Yadav and Yadav 2018b). Furthermore, they have also proved to be good biocontrol agents. Thus, these could be potent and novel alternatives to synthetic pesticides and chemical fertilizers, and such beneficial microbes are perfect candidates for sustainable agricultural production (Kumar et al. 2018; Palaniyandi et al. 2013).

Thus, from past few decades, there has been a strong upsurge of fungal community whether it may be in the agricultural sector or in the spheres of food, feed, and therapeutics. Adding more, their biocatalytic potential has been utilized for centuries for production of bread, wine, vinegar, and many more products (Fig. 1.1). Even the first report of commercial application of yeast for production of alcoholic beverages from barley was by the Babylonians and Sumerians as early as 6000 BC (Biswas et al. 2018; Singh et al. 2016a; Yadav et al. 2018c). Adding more, microbes are favored sources for industrial enzymes among which fungi are in fact attractive producers of diverse enzymes as they are easily available and due to their fast

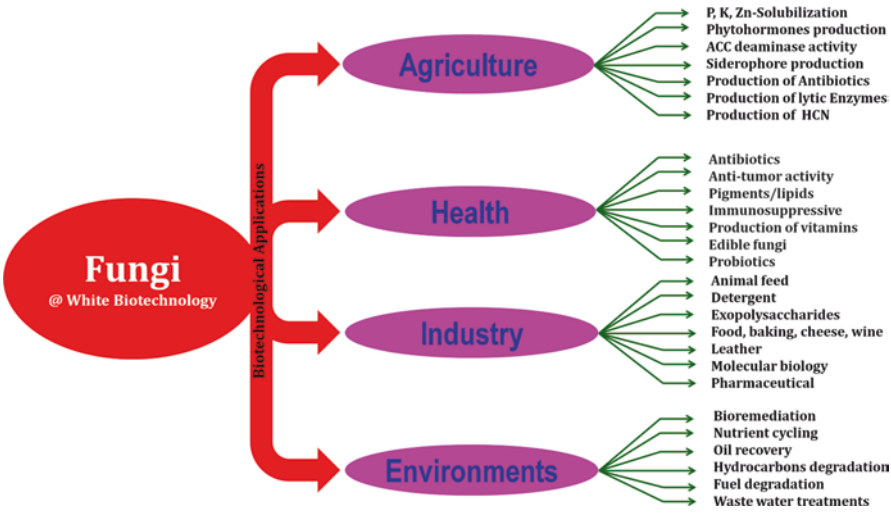

Fig. 1.1 Biotechnological applications of fungi and their value-added products in agriculture, health, industry, and environments

growth rate. Furthermore, genetic changes in microbial cells for elevated production of enzymes using recombinant DNA technology are also easy (Illanes et al. 2012; Singh et al. 2016a). Therefore, keeping in view the importance of the fungal communities in the agriculture, industry and allied sectors, the present chapter deals with the impact of fungi in agriculture and fungal enzymes utilized in diverse industrial and allied sectors.

1.2 Beneficial Impact of Fungal Communities in Agriculture

Plant growth-promoting fungi are gaining significant interest to be used as bioinoculants as they possess manifold benefits on the quantity as well as quality of the plants and because of the positive relation they exhibit with the ecological environment. Though most of the work on plant growth-promoting microbes (Verma et al. 2015a, b, c, 2016a) is focused on bacteria as well as mycorrhizal fungi (Johansson et al. 2004; Kumar et al. 2018), fungi still possess certain characteristic features which are far superior to bacteria, for example, fungi are able to tolerate acidic conditions better and they are in fact far better in mobilizing bound phosphates over bacteria (Kumar et al. 2018; Wahid and Mehana 2000). Furthermore, fungi have been demonstrated to produce phytohormones including indole-3-acetic acid (IAA), gibberellins (Kumar et al. 2018), and siderophores (Kumar et al. 2018; Milagres et al. 1999) (Fig. 1.2). Thus, this section deals with the role of fungi in plant growth promotion.

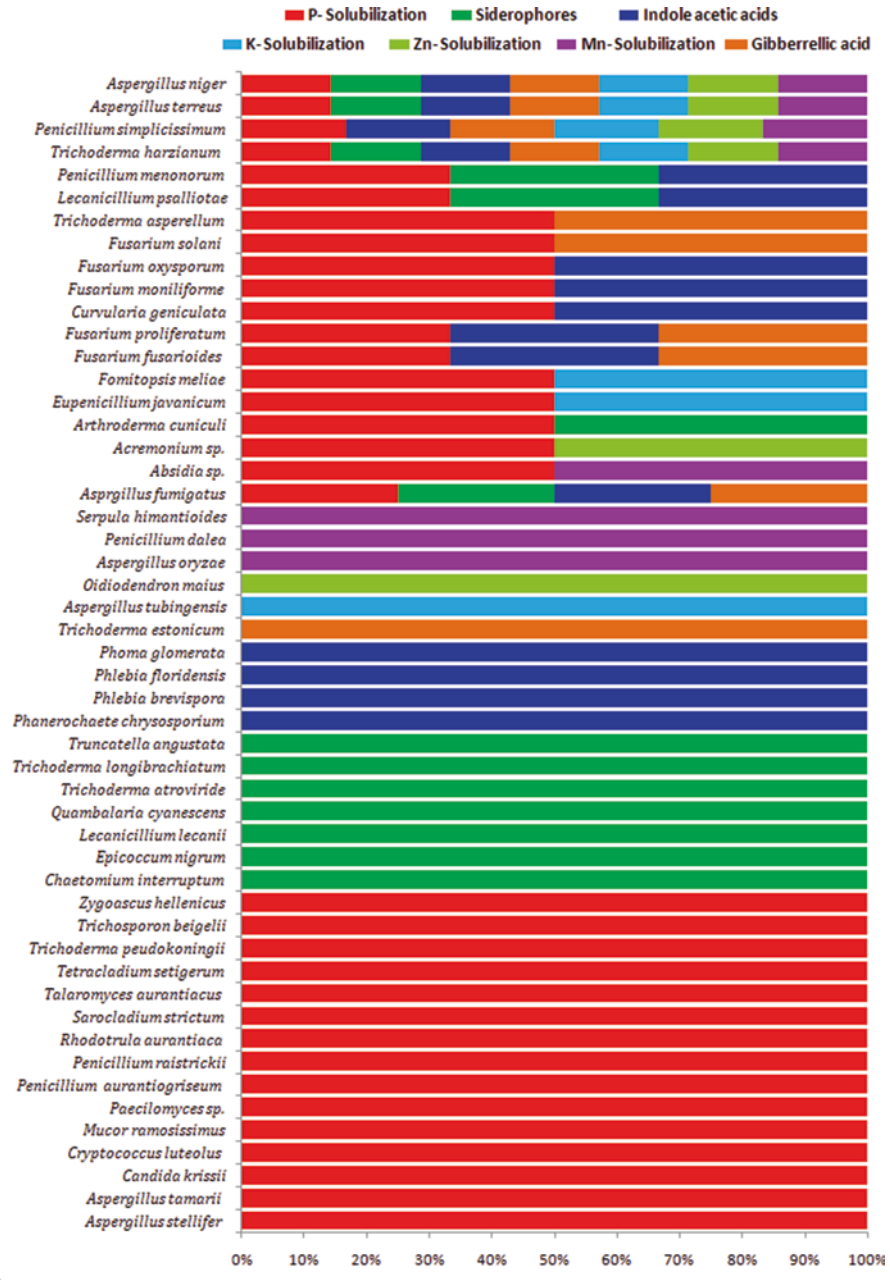

Fig. 1.2 Multifunctional plant growth attributes of fungal community

1.2.1 Nutrients Acquisition and Plant Growth

The utilization of microbial-based approaches is surely going to be novel alternative for reducing the environmental pollution which is on rise due to excessive use of chemical fertilizers so as to meet the nutrient requirements of the plants (Yadav 2017; Yadav et al. 2018a; Yadav and Yadav 2018b). Soil harbors a range of microorganisms, among them fungi are one of the members of mixed community categorized by complex interactions. The interactions between the plants and the microbes in the rhizospheric region are one of the key determinants of health of the plants as well as the fertility of soil (Anwar et al. 2014; Karmakar et al. 2018; Verma et al. 2017b; Yadav et al. 2016c). Fungi play an important role in making available various nutrients such as phosphorus, iron, zinc, manganese and potassium to the plants which are usually unavailable.

1.2.1.1 Phosphorus Acquisition and Plant Growth

Phosphorus is one of the vital macronutrients next to nitrogen important for growth and development of plants (Hameeda et al. 2008; Karmakar et al. 2018). But about 95–99% of soil phosphorus is present in insoluble form complexes with cations such as aluminum, calcium, and iron and cannot be utilized by the plants (Karmakar et al. 2018; Son et al. 2006). Thus, to fulfill the phosphorus requirements of the plants, phosphatic fertilizers are used, but chemical fertilizers are not eco-friendly, and thus there becomes a need for some eco-friendly alternate strategies to reduce the use of chemical fertilizers. There are diverse microbes including bacteria, fungi, and actinomycetes in soil which possess capability to solubilize phosphorus by producing organic acids such as alpha-ketobutyric acid, citric acid, fumaric acid, gluconic acid, glyoxylic acid, 2-ketogluconic acid, malic acid, oxalic acid, succinic acid, and tartaric acid (Yadav and Saxena 2018; Yadav et al. 2015a, 2017b, f). Furthermore, phosphatases are also known to play a chief role in transforming organic forms of phosphorus into plant available inorganic forms (Pandey et al. 2008; Yadav et al. 2016a). Phosphorus-solubilizing microbes have been reported from different environmental niches (Gaba et al. 2017; Saxena et al. 2016; Singh et al. 2016b). Fungi have been reported to exhibit greater capability to solubilize insoluble phosphate as compared to bacteria (Nahas 1996; Pandey et al. 2008; Yadav 2018).

Furthermore, there are reports on application of phosphorus-solubilizing fungi to crops which has been revealed to enhance the yield. Mittal et al. (2008) evaluated the impact of six phosphate-solubilizing fungi including two strains of *Aspergillus awamori* and four of *Penicillium citrinum* on the growth and seed production of *Cicer arietinum* in pot experiment under greenhouse conditions. The inoculation resulted in increase in the shoot height, seed number, and seed weight in inoculated plants though the increment in the studied parameters was found to be higher in inoculation with *Aspergillus awamori* strains. Kapri and Tewari (2010) evaluated

the effect of inoculation with P-solubilizing *Trichoderma* sp. on *Cicer arietinum* and found out that the inoculation with the P-solubilizing strain increased all the growth parameters studied which included fresh and dry weight of shoot as well as roots, shoot and root length in P-deficient soil containing only bound phosphate (TCP). Promwee et al. (2014) observed that the inoculating *Hevea brasiliensis* with phosphorus-solubilizing *Trichoderma harzianum* along with rock phosphate increased leaf number, plant height, stem circumference, shoot fresh weight, root fresh weight, shoot dry weight, and root dry weight as well as total phosphorus in leaves, as compared with the control consisting of only rock phosphate.

The study on the combined effect of phosphorus-solubilizing *Bacillus* sp. and *Aspergillus niger* on growth and yield of *Cicer arietinum* has been done under a pot experiment (Saxena et al. 2015b). The results indicated that the overall growth of the plants was better with dual inoculation as compared to uninoculated control and single inoculations. Priyadharsini and Muthukumar (2017) studied the effect of inoculating *Cajanus cajan* with *Curvularia geniculata* and observed that inoculated plants showed better growth in comparison to uninoculated control plants. The study revealed that fungus mediated the growth through solubilization of phosphorus as well the production of IAA and concluded that the mechanism used by fungus for plant growth promotion would enable the use of this fungus as bioinoculant in plant production systems. Zhou et al. (2018) observed in greenhouse experiment that *Alternaria* sp. (A13) at the same time enhanced the dry root biomass along with secondary metabolite accumulation of *Salvia miltiorrhiza*. Further, the seedlings of *Salvia miltiorrhiza* colonized by *Alternaria* sp. showed noteworthy increase in fresh weight and dry weight, as well as enhancement in the contents of total phenolic acid, lithospermic acids A and B (LAA and LAB, respectively), respectively. The study finally concluded that *Alternaria* sp. (A13) not only contributed to the stimulation of *Salvia miltiorrhiza* root growth but also boosted up the secondary metabolism, thus suggesting its application potential as a biofertilizer for *Salvia miltiorrhiza* cultivation, especially in areas outside of its native growth regions.

Thus, phosphorus-solubilizing fungi could be utilized to make use of the fixed P in soil for the crops without causing any harm to the environment. Though there is a large number of phosphorus-solubilizing microbes in soil, their application to the crops is still limited and there is a need to explore more of them so that they could be used as biofertilizers in sustainable crop production.

1.2.1.2 Potassium Acquisition and Plant Growth

Potassium is the third most important macronutrient plays a chief role in the process of photosynthesis and synthesis of proteins and enzymes, provides resistance to disease and insects, regulates permeability of cell membranes, and keeps the protoplasm in a proper degree of hydration; further it also plays an integral part in the development of chlorophyll (Meena 2016; Verma et al. 2016a, 2017a). The deficiency of potassium leads to stunted growth with shortening of internodes; reduction of photosynthesis; blackening of tips or margin of lower leaves of legumes,

maize, cotton, and tobacco and tubers in the case of potato; and either scorching or burning of all small grains (Ashley et al. 2005; Meena 2016).

Potassium is present in abundance in soil or is also applied to fields either as natural or as synthetic fertilizers, but the availability of potassium to plants is just 1–2% and the rest remains bound to other minerals. The most common soil components of potassium, 90–98%, are feldspar and mica (McAfee 2008; Meena 2016). Due to unavailability of potassium to plants, there comes the need of applying the fertilizers. Inorganic fertilizers are not directly toxic to humans and other life forms, but their presence completely disturbs the existing ecological balance, and the contamination of the environment arises just because all the fertilizers which are applied are not taken up by the crop and removed at the harvest (Meena 2016). But, the use of potassium fertilizers has not solved the problem especially in developing countries such as India as it lays a major economic constraint because a large amount of money is spent just on potassium fertilizers alone. In order to combat this problem, there are diverse groups of microbes in soil which have been reported to solubilize insoluble as well as the fixed forms of potassium which are then easily absorbed by the plants (Gundala et al. 2013; Meena 2016). The mobilization of potassium is greatly affected by various abiotic as well as biotic factors including the properties of soil such as pH, aeration, physicochemical characteristics, and presence of AMF, fungi, and bacteria and composition of the root exudates (Meena 2016). AMF increases the solubility of mineral form of potassium by releasing protons, H^+, or CO_2 and organic acid anions, for instance, malate, oxalate, and citrate.

Wu et al. (2005) evaluated the effects of arbuscular mycorrhizal fungus including *Glomus mosseae* or *Glomus intraradices* with or without nitrogen fixer; *Azotobacter chroococcum*, phosphorus solubilizers; *Bacillus megaterium* and potassium solubilizers; and *Bacillus mucilaginous* on soil properties and the growth of maize. The application of biofertilizer containing mycorrhizal fungus and three species of bacteria considerably increased the growth of maize. The application of *Glomus mosseae* and *Azotobacter chroococcum, Bacillus megaterium*, *Bacillus mucilaginous* resulted in the highest biomass and seedling height. The results revealed that the inoculum increased total N, P, and K, also further improving organic matter content and total N in soil. Gore and Navale (2017) isolated 19 isolates from the rhizospheric soils of Maharashtra, among which 3 fungal isolates were found to be best solubilizers of mica. All the three isolates were identified as *Aspergillus niger* with isolate KSF 3 showing maximum solubilization, i.e., 334.66 mg l^{-1} at 10 days after incubation.

1.2.1.3 Zinc Acquisition and Plant Growth

Zinc is another vital micronutrient for growth of plant. It is an important constituent of a variety of metabolic enzymes. Due to its poor mobility in plants, there is a need for a constant supply of available zinc to fulfill demands of the plants (Saravanan et al. 2007; Yadav et al. 2018b). Therefore, zinc is made available to the plants in the form of the fertilizers, one of them being zinc sulfate which in turn gets transformed

into diverse insoluble forms. This transformation depends upon certain factors such as type of soil and chemical reaction taking place in soil and is totally unavailable within 7 days of application in the environment (Rattan and Shukla 1991; Saravanan et al. 2007). Fungi have a chief role in biogeochemical change of the mineral rocks as well as an important role in mobilizing the insoluble compounds (Sutjaritvorakul et al. 2017). Thus, fungi can enhance the bioavailability of zinc to the plants. Fungi actually produce organic acids which enhance the mobilization of zinc present in the insoluble form to readily available form in soil solution (Adeyemi and Gadd 2005; Sutjaritvorakul et al. 2017; Yadav et al. 2018d). A few fungal genera have been known which show capacity to solubilize insoluble zinc compounds such as *Abisidia cylindrospora*, *Abisidia glauca*, *Abisidia spinosa*, *Aspergillus niger*, and *Penicillium simplicissimum* (Coles et al. 1999; Saravanan et al. 2011). Ericoid mycorrhizae including *Beauveria caledonica*, *Hymenoscyphus ericae*, *Oidiodendron maius*, *Paxillus involutus*, *Suillus luteus*, and *Suillus bovinus* have also been known to solubilize insoluble zinc compounds (Saravanan et al. 2011). Martino et al. (2003) demonstrated the solubilization of insoluble inorganic zinc compounds by *Oidiodendron maius*. Sutjaritvorakul et al. (2013) identified *Aspergillus niger*, *Aspergillus* sp., and *Phomopsis* sp. from zinc-containing rocks and mining soil as solubilizers of insoluble zinc compounds. It was found that about 87% of the tested fungi solubilized zinc oxide, whereas 61% and 52% solubilized zinc carbonate and zinc phosphate, respectively. Sutjaritvorakul et al. (2017) showed *Aspergillus niger* and *Aspergillus* sp. as solubilizers of zinc oxide nanoparticles which were isolated from zinc sulfide mineral ores.

1.2.1.4 Manganese Acquisition and Plant Growth

Another vital nutrient for the plants is manganese. It plays an important role in photosynthesis, formation of chloroplast, nitrogen metabolism, and synthesis of some enzymes. Further, it is among the most plentiful metals on the earth's crust (Sinha and Khare 2013). The deficiency of manganese is one of the major problems mostly known to occur in sandy soils, organic soils, and heavily weathered, tropical soils further deteriorated by cool and wet conditions (Alloway 2008). The deficiency of Mn leads to low production of dry matter, low yield, high susceptibility to pathogens, and reduction of tolerance to abiotic stress conditions such as drought, heat, etc. On the contrary, if Mn is present in plant tissues in excess, it alters diverse processes including absorption, translocation, and utilization of calcium, magnesium, iron, and phosphorus, enzyme activity, and so on (Lei et al. 2007; Millaleo et al. 2010). Mn mostly occurs in the form of minerals such as carbonates, oxides, phosphorus, pyrophosphates, sulfides, etc. (Sinha and Khare 2013). In fact, the biochemistry of Mn is rather complex in soil due to its presence in diverse oxidation states including 0, II, III, IV, VI, and VII (Millaleo et al. 2010). Diverse factors such as pH and redox conditions greatly influence Mn bioavailability in soils (Millaleo et al. 2010; Porter et al. 2004). In acidic soils and increased redox potential of Mn, oxides are easily reduced in soil exchange sites (Kogelmann and Sharpe 2006),

which increases the concentration of soluble Mn^{2+} (Watmough et al. 2007), which is actually the principal form of Mn in the soil solution (Adriano 2001) and the most available form of manganese to the plants (Millaleo et al. 2010). On the other hand, at higher pH chemical Mn^{2+} auto-oxidation is favored over MnO_2, Mn_2O_3, Mn_3O_4, and even Mn_2O_7, which are not usually available to plants (Ducic and Polle 2005; Millaleo et al. 2010). Additionally, high pH supports the adsorption of manganese into soil particles further reducing its availability (Millaleo et al. 2010). In soil, nutrients undergo a complex dynamic equilibrium of solubilization and insolubilization that is significantly influenced by the soil pH as well as microflora ultimately affecting accessibility to plant roots for absorption. A number of microorganisms, particularly those in association with roots, possess capability to enhance plant growth and productivity (Altomare et al. 1999; Kloepper et al. 1988; Srivastava et al. 2013). In some cases, this effect has been known to involve solubilization of unavailable mineral nutrients (Goldstein 1995).

Altomare et al. (1999) evaluated the capability of plant growth-promoting and biocontrol fungus *Trichoderma harzianum* to solubilize in vitro some insoluble or sparingly soluble minerals. The strain solubilized MnO_2, metallic zinc, and rock phosphate. Wei et al. (2012) demonstrated the capability of *Aspergillus niger* and *Serpula himantioides* to solubilize all the insoluble oxides when incorporated into solid medium: MnO_2 and Mn_2O_3, mycogenic manganese oxide, and birnessite. Mohanty et al. (2017) isolated *Aspergillus terreus*, *Aspergillus oryzae*, *Penicillium dalea*, and *Penicillium* sp. as solubilizers of manganese from low-grade manganese mine tailings.

1.2.2 Bioprotection Using Fungal Communities

Use of pesticides in agriculture has led to groundwater as well as environmental pollution concerns, and further due to lack of efficient chemical agents, biological control agents are one of the most potent alternative approaches for controlling plant pathogens so that the use of pesticides could be reduced (Akhtar and Siddiqui 2008; Dolatabad et al. 2017). Fungi are among one of the novel and potential sources of biological control agents.

The study of Brum et al. (2012) showed potential antagonistic activity of *C. gloeosporioides* and *Flavodon flavus* against *Fusarium oxysporum* f. sp. *herbemontis*. Erler and Ates (2015) showed the effectiveness of *Beauveria bassiana* and *Metarhizium anisopliae* against the larvae of *Polyphylla fullo*. Yuan et al. (2017) evaluated the role of *Acremonium* sp. (CEF-193), *Leptosphaeria* sp. (CEF-714), *Penicillium simplicissimum* (CEF-818), and *Talaromyces flavus* (CEF-642) on *Verticillium* wilt of cotton caused by defoliating *V. dahliae* (Vd080) in a greenhouse experiment. It was found that all the treatments reduced the incidence of disease especially strains CEF-818 and CEF-714 which provided protection well. The treatment with *Penicillium simplicissimum* appreciably increased the seed cotton yield. *Penicillium simplicissimum* (CEF-818) and *Leptosphaeria* sp. (CEF-

714) also increased transcript levels for PAL, PPO, and POD, which actually leads to the increase of cotton defense reactions. Finally, the study suggested that seed treatment of cotton plants with strains CEF-818 and CET-714 can help in the biocontrol of *V. dahliae* and improve seed cotton yield in cotton fields. In another study of Saravanakumar et al. (2017), the effect of *Trichoderma harzianum* (CCTCC-RW0024) on changes of maize rhizosphere microbiome and biocontrol of *Fusarium* stalk rot caused by *Fusarium graminearum* was investigated. The study revealed that the strain displayed high antagonistic activity, disease reduction, and biocontrol-related enzyme and gene expression and concluded that *Trichoderma harzianum* (CCTCC-RW0024) is an effective biocontrol agent against *Fusarium* stalk rot. Contreras-Cornejo et al. (2018) investigated the effects of *Trichoderma atroviride* in providing *Zea mays* resistance against the insect herbivore, *Spodoptera frugiperda*. The study observed increase in plant growth, reduction in herbivory, as well as altered feeding patterns after inoculating maize with *Trichoderma atroviride* and correlated plant protection with increase in the emission of volatile terpenes and accumulation of jasmonic acid. This section deals with diverse mechanisms used by fungi to provide protection to plants against pathogens.

1.2.2.1 Production of Siderophores

Iron is known to be the fourth most abundant element on the earth's crust and is vital for the growth and developmental processes of every living organism. It regulates the biosynthesis of antibiotics, aromatic compounds, cytochromes, nucleic acids, pigments, porphyrins, siderophores, toxins, and vitamins (Saha et al. 2016). Iron plays an important role in a number of metabolic processes such as electron transport chain, oxidative phosphorylation, photosynthesis, and tricarboxylic acid cycle (Yadav et al. 2017c, d). Recently, it has also been known to play an important role in the formation of the microbial biofilm where it regulates the surface motility of microorganism (Cai et al. 2010; Glick et al. 2010; Saha et al. 2016; Yadav et al. 2017e, f). Iron exists in two states in aqueous solution including Fe^{2+} and Fe^{3+}, but Fe^{3+} forms cannot be utilized by the plants as well as the microorganisms as they form oxides and hydroxides which are insoluble, in turn limiting the bioavailability (Desai and Archana 2011; Zuo and Zhang 2011). Microbes have developed numerous ways for iron scavenging, one of these being siderophore-mediated acquisition of iron through specific receptor and transport system. Siderophores are low-molecular-weight, high-affinity ferric ion chelators that are excreted under iron starvation by diverse microbes such as bacteria and fungi and have also been known to be excreted by some plants. Generally, iron is acquired when excreted siderophores bind with the available ferric ion which forms ferri-siderophore complex, and this complex ultimately binds to the specific receptor protein that is present on microbial cell surface. The complex is translocated by active transport and is released inside the cell (Khan et al. 2017). Roots can also take up iron from this ferri-siderophore complex by diverse ways such as chelate degradation, direct uptake of the complex, and ligand exchange reaction.

Siderophores of fungal origin are high-affinity iron-chelating, linear to cyclic oligomeric secondary metabolites (Renshaw et al. 2002; Speckbacher and Zeilinger 2018). Siderophores are important metabolites in the response against oxidative stress in several fungi like *Trichoderma virens*, *Gibberella zeae*, *Cochliobolus heterostrophus*, *Aspergillus fumigatus*, and *Aspergillus nidulans*, furthermore playing an important role in conidial germination and sexual development (Oide et al. 2007; Speckbacher and Zeilinger 2018). There are different types of siderophores, and each one differs from one another in their chemical structure and properties. On the basis of chemical structure, siderophores have been categorized as carboxylate, catecholate, and hydroxamate types. Fungi are known to produce generally carboxylate and hydroxamate type of siderophores. The most extensively studied for the production of the siderophores include *Aspergillus fumigatus* and *Aspergillus nidulans* having 55 similar types of siderophores (Khan et al. 2017).

The production of the organic acids by fungi is possibly the reason that fungi produce hydroxamate type of the siderophores, which are stable down to pH 2 (Szebesczyk et al. 2016; Winkelmann 2002). In fungi these are hydroxylated and alkylated ornithine based (Baakza et al. 2004; Khan et al. 2017). It consists of N^5-acyl-N^5-hydroxyornithine or N^6-acyl-N^6-hydroxylysine (Winkelmann 2002). There are different types of hydroxamate type of the siderophores that are produced by fungi such as coprogens which are chiral linear hydroxamate siderophores. These were firstly isolated from a culture of the *Pilobolus* (Hesseltine et al. 1952; Szebesczyk et al. 2016) and are mostly produced by *Penicillium* sp. and *Neurospora crassa* (Szebesczyk et al. 2016). These have been also known to be produced by a number of phytopathogens and even by some human pathogens (Howard 1999). Another hydroxamate type of siderophore includes fusarinine known to be common in species of *Aspergillus*, *Fusarium*, *Gliocladium*, and *Paecilomyces* (Szebesczyk et al. 2016). In fact, *Aspergillus fumigatus* has been known to utilize fusarinine C and triacetyl fusarinine C for capturing extracellular iron (Khan et al. 2017). Further, ferrichromes present one of the largest families of hydroxamate-based siderophores. These are known to be produced by *Aspergillus* sp. and *Penicillium* sp. In fact by pathogen *Aspergillus fumigatus* (Howard 1999), certain phytopathogens are known to produce ferrichromes (Szebesczyk et al. 2016).

Nakamura et al. (2017) showed ASP2397, a novel antifungal agent from *Acremonium persicinum* MF-347833 similar to ferrichrome. Another siderophore of this category is rhodotorulic acid known to be produced by some yeasts, chiefly by the *basidiomycetous* yeasts (Atkin et al. 1970), and also by the smut fungi *Sphacelotheca* and *Ustilago* (Deml 1985). In fact, it was firstly isolated from culture of the *Rhodotorula pilimanae* (Atkin and Neilands 1968; Müller et al. 1985). In two ectomycorrhizal basidiomycetes, *Laccaria laccata*, common woodland fungus, and *Laccaria bicolor*, a model organism used in research, the principal siderophores have been reported which is the ester-containing siderophore linear fusigen in addition to coprogen, ferricrocin, and triacetylfusarinine C in small quantities (Haselwandter et al. 2013). There are only two non-hydroxamate siderophores that have been isolated and fully characterized including rhizoferrin isolated from

Rhizopus microsporus and pistillarin, produced by marine fungus *Penicillium bilaii* (Capon et al. 2007).

Siderophores are known to act as a potential biocontrol agent against harmful phyto-pathogens thus holding the capability to substitute hazardous pesticides. Segarra et al. (2010) studied the importance of iron concentration in the growth media for the activity and competitiveness of *Trichoderma asperellum* (T34) and pathogen (*Fusarium oxysporum* f. sp. *lycopersici*) on tomato plants using different concentrations of iron, and the study hypothesized that iron competition is one of the chief factors in the biocontrol activity exerted by *Trichoderma asperellum* (T34) against the pathogen. The study concluded that *Trichoderma asperellum* protected tomato plants from both *Fusarium* wilt and abiotic stress, i.e., toxic effects of Fe (III). In the study of Sun et al. (2017), *CmSIT1* gene which is involved in the siderophore-mediated iron transport has been cloned as well as functions have been studied in mycoparasite of *S. sclerotiorum*, i.e., *Coniothyrium minitans*. The study revealed that the expression of this gene led to reduction of growth simultaneously enhancing the antifungal capability. The antifungal substances appreciably inhibited the infection of *S. sclerotiorum* on the leaves of rapeseed. Mukherjee et al. (2018) studied the role of intracellular siderophores in *Trichoderma*-plant interactions for which authors obtained mutants in a non-ribosomal peptide synthetase, TvTex10, which was predicted to be involved in intracellular biosynthesis of siderophores. The study concluded that mutants were impaired in inducing induced systemic resistance in maize against the foliar pathogen *Cochliobolus heterostrophus*.

1.2.2.2 Production of Antibiotics

Soil is very complex, with several constituents each one performing different functions chiefly due to the activity of soil organisms (Al-Enazi et al. 2018; Ullah et al. 2017). The microorganisms play an important role in soil ecosystem. The quality of soil is determined by its microbial composition and functioning. Fungi are very important for the soil ecosystem and play a considerable role in the daily life of human beings additionally important for agriculture, bioremediation, natural cycling, food industry, as bio-fertilizers (Karthikeyan et al. 2014; Yadav and Yadav 2018a). Further, fungi are also an important source of secondary metabolites. Ecologically, soils have been considered to be fertile sources of antibiotic-producing microbes due to strong competition for nutrients and territory in this microbially rich habitat. The screening of antibiotics started with organisms from soils over 60 years ago, and a high percentage of known secondary metabolites from fungi have been obtained from soil isolates. The production of secondary metabolites for instance antimicrobial agents is one of the most important uses of fungi which can actually be beneficial for medical therapy (Al-Daamy et al. 2018; Farjana et al. 2014). Secondary metabolites are referred to as small organic molecules produced by an organism which are not necessary for their growth, development, and

reproduction; rather they play an important role in antagonism, competition, and self-defense mechanisms against other living organisms so as to allow the organism to occupy the niche and utilize the food. Fungi produce many antibiotics, exhibiting antibacterial and antifungal activities, respectively, which are widely used as drugs over the world especially the cephalosporin and fusidic acid and penicillin (Al-Enazi et al. 2018). Furthermore, endophytes of vascular plants are the most extensively explored ecological group during the past years (Karwehl and Stadler 2016). Fungal endophytes have been reported to produce novel antibacterial, anticancer, antifungal, anti-inflammatory, anti-malarial, and antiviral substances (Higginbotham et al. 2013; Supaphon et al. 2018; Wiyakrutta et al. 2004).

Al-Daamy et al. (2018) screened the filtrates of *Aspergillus flavus*, *Aspergillus niger*, *Aspergillus ochraceus*, *Bacillomyces* sp., *Cladosporium* sp., *Penicillium notatum*, and *Trichoderma* sp. for their antimicrobial activity against *Bacillus* sp., *Enterobacter* sp., *Klebsiella* sp., *Pseudomonas* sp., *Staphylococcus* sp., and *Streptococcus agalactiae* by disc technique. The study revealed *Bacillus* sp. to be the most sensitive to all fungal filtrations. Awad et al. (2018) evaluated *Trichoderma viridae* as antimicrobial, antioxidant, and anticancer agent isolated from rhizospheric soil of cucumber. The study showed that *Trichoderma viride* caused the inhibition of the mycelial growth of *Fusarium solani*, *Rhizoctonia solani*, and *Sclerotium rolfsii*. Furthermore, alcoholic extract of the fungal mycelia exhibited a potent antibacterial activity against *Bacillus subtilis*, *E. coli*, and *Pseudomonas fluorescens* additionally also exhibiting considerable antifungal activity against *Candida albicans*, *Fusarium solani*, *Fusarium oxysporum*, *Rhizoctonia solani*, and *Pythium ultimum*. Ribeiro et al. (2018) showed inhibitory activity of extracts of *Curvularia* sp. and *Diaporthe* sp. against pathogenic Gram-negative as well as Gram-positive bacteria including *E. coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Staphylococcus epidermidis*.

1.2.3 Biostimulation

The biostimulation approach uses microbial inoculants, biofertilizers, biochemicals, and organic amendments for a long time to get better soil health, fertility, plant productivity, and soil remediation. Biostimulation is a technique that relies on increasing the activity of the indigenous microbes by adjusting the factors that may limit their activity, mainly oxygen and nutrients. In another term, biostimulation involves the application of a proper agent to soil to enhance the activity of indigenous microorganisms (Kour et al. 2017a, b; Malina and Zawierucha 2007). The word biostimulant was first defined by Kauffman et al. (2007) according to which "biostimulants are materials, other than fertilizers, that promote plant growth when applied in low quantities." Kauffman et al. (2007) generally classified biostimulants into three major groups which include humic substances (HS), hormone-containing products (HCP), and amino acid-containing products (AACP). Industry is a key

player in the promotion of the concept of biostimulants, including microorganisms. The companies, corporate sector created associations and also supported the organization of first international symposiums in November 2012, the "First World Congress on the use of biostimulants in agriculture," which took place in Strasbourg regarded as a milestone into the academic area.

The natural constituents of the soil organic matter, resulting from the decomposition of plant, animal, and microbial residues, by action of soil microbes are referred to as humic substances (HS) (Eyheraguibel et al. 2008). For the long time, humic substances are recognized to improve the physical, chemical, and biological properties of soil by improving its fertility. Humic substances also play activity in stress protection. High-molecular-mass HS phenolic compounds are involved in a wide range of stress responses (Olivares et al. 2015; Schiavon et al. 2010). By chemical and enzymatic method, amino acids are obtained from agro-industrial by-products, plant sources, and animal wastes (Calvo et al. 2014; Du Jardin 2012). These compounds have been shown to play multiple roles as biostimulants of plant growth (Calvo et al. 2014). Hormonal activities are also reported in complex protein and tissue hydrolysates (Colla et al. 2014). Protein hydrolysates are known to increase microbial biomass and activity, soil respiration, and soil fertility.

The interaction of fungi with plant roots occurs in different ways, from mutualistic symbioses to parasitism (Behie and Bidochka 2014). Over 90% of all plant species mycorrhizal fungi establish symbioses arbuscule-forming mycorrhiza (AMF), where fungal hyphae penetrate root cortical cells and form branched structures called arbuscules (Behie and Bidochka 2014; Bonfante and Genre 2010). Application of fungal-based products when applied to plants if promote the nutrition effectiveness, easiness to stress, crop productivity, etc. should fall under the concept of biostimulants (du Jardin 2015). Endophytic fungi like *Trichoderma* sp. and *Piriformospora indica* reported to colonize the roots of plants and transfer nutrients to their hosts (Behie and Bidochka 2014). In the biotechnological industries, *Trichoderma* spp. have been used for their biopesticidal and biocontrol capacities and are also source of enzymes. These fungal endophytes may be regarded as biostimulants (Mukherjee et al. 2013; Nicolas et al. 2014).

The phytohormones such as auxins, cytokinins, gibberellic acid, abscisic acid, and salicylic acid are signal molecules produced within plants in extremely low concentrations. Some phytohormones also occur in microorganisms, such as unicellular fungi and bacteria. Organic compounds of phytohormones, except for nutrients, are called biostimulants. Auxins are indole-derived hormones which are involved in plant developmental processes, such as cell division, differentiation and organ formation and senescence further also control biotic and abiotic stress responses in plants (Benjamins and Scheres 2008; Kim et al. 2011; Okal et al. 1999). Auxins hormones could have an endogenous role in many fungal species (Gruen 1959; Ulrich 1960). Auxins are involved in symbiotic interactions between plants and bacteria or fungi. They are also vital for the beginning of nodule formation in the nitrogen-fixative bacterial symbiosis (Hirsch and Fang 1994) and for the invasion of mycorrhizal fungi (Etemadi et al. 2014).

The cytokinins are well known for developmental processes in plant, for instance, development of root and shoot, cell differentiation, and delay of senescence (Barciszewski et al. 1999; Peleg et al. 2011). Fungal species whether saprophytic, pathogenic, or symbiotic are known for their ability to produce CKs (Cooper and Ashby 1998; Murphy et al. 1997). Chanclud et al. (2016) in his study reported that endogenous and exogenous CKs are required for oxidative stress tolerance in the rice blast fungus *Magnaporthe oryzae*. Gibberellic acid was for the first time produced by *Gibberella fujikuroi*, the fungus which causes "foolish seedlings" disease of rice. GAs play an important role in the control of germination, flowering, cell division, and internode elongation (Brian et al. 1954; Lange and Lange 2006; Swain and Singh 2005). ABA is the key hormone for plant abiotic stress responses (Peleg and Blumwald 2011). The abscisic acids provide drought tolerance in plants along with stomatal closure (Beardsell and Cohen 1975). In *Cercospora risicola* firstly production of abscisic acid was known (Norman et al. 1983). The abscisic acid in *Magnaporthe oryzae* reported to increase the germination rate and the development of appressoria (Spence et al. 2015).

Certain literature reported fungi can also produce phytohormones which can promote the growth and development of plant and also induces acceptance against various environmental stress factors (Priyadharsini and Muthukumar 2017). Phytohormones play a significant role in symbiotic associations of plants with arbuscular mycorrhizae and rhizobial. The plant hormones secreted by plant as strigolactones initiate the growth of arbuscular mycorrhizae and attract them toward the roots (Gutjahr 2014). Waqas et al. (2012) isolated and examined endophytic fungi *Phoma glomerata* LWL2 and *Penicillium* sp. LWL3 significantly promoted the growth of GAs-deficient dwarf mutant Waito-C and Dongjin-beyo rice by secretion of phytohormones viz. gibberellins (GAs) and indoleacetic acid (IAA). Lei and Zhang (2015) reported in their study that *Trichoderma asperellum* Q1 exhibited the ability to increase the levels of three phytohormones in cucumber seedling indoleacetic acid (IAA), gibberellic acid (GA), and abscisic acid (ABA).

Barea and Azcón-Aguilar (1982) studied *Glomus mosseae* which forms vesicular-arbuscular mycorrhiza for phytohormone production. Paper partition chromatography and specific bioassays specified microorganism synthesized at least two gibberellin-like substances, one with *Rf* corresponding in position to authentic gibberellic acid, and four substances with the properties of cytokinins. Khan et al. (2011) identified a new strain of *Aspergillus fumigatus* as an endophytic fungus, which is little known for exogenous gibberellins (GAs) production. This novel endophytic fungus has reprogrammed soybean metabolism to improve plant growth and increase isoflavone contents under salt stress. The phytohormones, abscisic acid and cytokinin, previously were considered to be present exclusively in plants, but rising verification support that these phytohormones are found in a various organisms. Few findings have examined fungi for the existence of these "plant" hormones. Twenty temperate forest fungi of differing nutritional modes (ectomycorrhizal, wood-rotting, saprotrophic) were studied. The study indicated fungi have the capacity to synthesize abscisic acid and cytokinin, these two classes of phytohormones (Morrison et al. 2015).

1.3 Fungi and Fungal Enzymes in Industrial Processes

Enzymes from microbes have gained great appreciation worldwide for their extensive uses in a variety of sectors whether it is food industry, agriculture, chemical industry, or medicine. In the field of medicine, these are used to treat health disorders associated with deficiency of human enzymes caused by genetic problems (Anbu et al. 2015; Singh et al. 2016a). The processes mediated by enzymes are speeding up in the food industry, pharmaceutical industry, textile industry, paper industry, and leather industry and are gaining interest because of certain advantages such as reduced process time, intake of low energy input, cost-effectiveness, greater efficiency, nontoxicity, higher-quality products, and eco-friendly characteristics (Gurung et al. 2013; Kamini et al. 1999; Singh et al. 2016a) (Table 1.1). The production of microbial enzymes at industrial level is essential due to high and better performances of enzymes from diverse microbes that work under a wide range of chemical as well as physical conditions. Furthermore, as per the requirement of industries, microbial enzymes can be cultured chiefly by gene manipulations. This section describes about diverse fungal enzymes which are used in different industries for different purposes (Fig. 1.3).

1.3.1 Baking Industry

Baking is a common name which is used for the production of baked goods, which have wheat flour as its chief ingredient and key source of enzyme substrates for the product (Van Oort 2009; Miguel et al. 2013). The baked products include bread, cake, cookies, crackers, pastries, pies, and tortillas. Baked goods such as gluten-free products or rye bread are also included in baked products (Miguel et al. 2013). The development of bread process was a significant event in mankind. After the nineteenth century, with the advancements in agricultural mechanization, the quality of the bread was greatly improved and then white bread became a product within almost everyone's reach (Dupaigne and Westbrook 1999). But the major aspect which made a great contribution to evolution of the baking market was the introduction of industrial enzymes in the baking process (Miguel et al. 2013). The process of baking makes use of enzymes from three diverse sources including the endogenous enzymes in flour, enzymes which are associated with the metabolic activity of the dominant microorganisms, and exogenous enzymes which are added in the dough (Di Cagno et al. 2003). Diverse microbial enzymes are used in the baking industry, but this section will focus on fungal enzymes used in the baking industry.

Hemicellulases are a class of enzymes that involves the hydrolysis of hemicelluloses, which are a group of polysaccharides consisting of arabinoxylan, arabinogalactan, xylan, and xylobiose (Shallom and Shoham 2003). This group consists of an important enzyme, i.e., xylanase also known as endoxylanase and initially termed as pentosanase (Collins et al. 2005). Xylanases are glycosidase catalyzing the

Table 1.1 Industrial applications of fungal enzymes

Enzymes	Function	Fungus	References
Dairy industry			
Acid proteinase	Milk coagulation	*Aspergillus* sp.	Vishwanatha et al. (2010)
Lipase	Faster cheese ripening, flavor customized cheese	*Aspergillus niger*, *A. oryzae*	Neelakantan et al. (1999)
Lactases	Milk for people with milk tolerance problems especially infants	*Aspergillus niger, A. oryzae*	Smart et al. (1985)
Catalase	Significant improvement in the quality of cheese	*Aspergillus niger*	Saxena et al. (2001)
Baking industry			
Xylanase	Dough conditioning	*Aspergillus niger*	Saxena et al. (2001)
Lipase	Dough stability and conditioning	*Aspergillus niger*	Saxena et al. (2001)
Glucose oxidase	Dough strengthening	*Aspergillus niger*, *Penicillium chrysogenum*	Saxena et al. (2001)
Proteases	Improvements in the aroma of bread	*Aspergillus oryzae*	Taylor and Richardson (1979)
Beverage industry			
Pectinase	Depectinization	*Aspergillus oryzae*, *Penicillium funiculosum*	Yamasaki et al. (1964)
Amylase	Used in brewing and fermentation industries, the laundry industry	*Aspergillus niger, Aspergillus oryzae*	El-Zalaki and Hamza (1979) Jin et al. (1998)
Glucose oxidase	Oxygen removal from beer	*Aspergillus niger*	Saxena et al. (2001)
Cellulase	Fruit liquefaction	*Aspergillus niger*, *Trichoderma atroviride*	Singh et al. (2016a)
Protease	Restrict haze formation	*Aspergillus niger*	Singh et al. (2016a)
Naringinase	Debittering	*Aspergillus niger, Aspergillus oryzae, A. usamii*	Bram and Solomons (1965), Kishi (1955)
Naringinase	Debittering	*Cochiobolus miyabeanus*	Ito and Takiguchi (1970)
Naringinase	Debittering	*Coniothyrium diplodiella*	Nomura (1965)

(continued)

Table 1.1 (continued)

Enzymes	Function	Fungus	References
Naringinase	Debittering	*Penicillium decumbens*	Fukumoto and Okada (1974)
Naringinase	Debittering	*Phanopsis citri*	Ito and Takiguchi (1970)
Naringinase	Debittering	*Rhizotonia solani*	Ito and Takiguchi (1970)
Naringinase	Debittering	*Rhizopus nigricans*	Shanmugam and Yadav (1995)
Detergent industry			
Amylase	Carbohydrate stain removal	*Aspergillus* sp.	de Souza (2010)
Lipase	Fat stain elimination	*Aspergillus oryzae*, *A. flavus*	Greenough et al. (1996)
Protease	Protein stain removal	*Aspergillus oryzae*	Vishwanatha et al. (2009)
Cellulase	Color clarification	*Aspergillus niger*	Kuhad et al. (2011)
Leather industry			
Neutral protease	Dehairing, soaking	*Aspergillus niger*, *A. flavus*	de Souza et al. (2015)
Lipase	Degreasing	*Aspergillus oryzae*, *A. flavus*	Singh et al. (2016a)
Amylase	Fiber splitting	*Aspergillus* sp.	Singh et al. (2016a)
Organic synthesis			
Lipase	Synthesis of pharmaceuticals, polymers, biodiesels, biosurfactants	*Aspergillus oryzae*, *A. flavus*	Singh et al. (2016a)
Laccase	Synthesis of an indamine dye, conducting polyaniline	*Coriolus hirsutus*	Baker et al. (1996) Karamyshev et al. (2003)
Laccase	Synthesis of 3-(3,4-dihydroxyphenyl)-propionic acid derivatives	*Pycnoporus cinnabarinus*	Ponzoni et al. (2007)
Laccase	Polymerization to functional polymers	*Pycnoporus coccineus*	Uyama and Kobayashi (2002)
Laccase	Oxidative coupling of 3-methyl 2-benzothiazolinone hydrazone and methoxyphenols	*Pyricularia oryzae*	Setti et al. (1999)

(continued)

Table 1.1 (continued)

Enzymes	Function	Fungus	References
Laccase	Synthesis of aromatic aldehydes	*Trametes versicolor*	Fritz-Langhals and Kunath (1998)
Laccase	Polymerization of bisphenol A	*Trametes villosa*	Uchida et al. (2001)
Laccase	Oligomerization of protein	*Trametes hirsuta*	Mattinen et al. (2006)
Laccase	Oxidation of sugars derivatives	*Trametes pubescens*	Marzorati et al. (2005)
Laccase	Cross-linking of recombinant proteins	*Pyricularia oryzae*	Suderman et al. (2006)
Laccase	Synthesis of 3,4-dihydro-7,8-dihydroxy-2*H*-dibenzofuran-1-ones	*Agaricus bisporus*	Hajdok et al. (2007)
Laccase	Synthesis of poly(catechin)	*Myceliophthora*	Kurisawa et al. (2003)
Waste management			
Extracellular enzymes	Dye degradation	*Trametes versicolor*	Libra et al. (2003)
Laccase	Reduction of the phenolic components in olive-mill wastewater	*Lentinula edodes*	Casa et al. (2003)
Ligninases, acid protease, end0-1,4-glucanas, exo-1,4-β-glucosidase, cellobiose oxidase	Degrade a wide variety of structurally diverse organic compounds, including a number of environmentally persistent organopollutants	*Phanerochaete chrysosporium*	Bumpus and Aust (1987)
Laccase	Biodegradation of different Malachite Green, Azure B, Poly R-478, Anthraquinone Blue, Congo Red, Xylidine	*Coriolus versicolor*	Levin et al. (2005)
Laccase	Biodegradation of Acid Blue 62, Acid Blue 40, Reactive Blue 81	*Cerrena unicolor*	Michniewicz et al. (2008)
Laccase	Biodegradation of Chicago Sky Blue, Poly B-411, Remazol Brilliant Blue R, Trypan Blue	*Daedalea quercina*	Baldrian (2004)
Laccase	Biodegradation of Reactive Black 5	*Funalia trogii*	Mazmanci and Ünyayar (2005)
Laccase	Biodegradation of Remazol Brilliant Blue Royal (RBBR), Drimaren Blue CL-BR	*Funalia trogii*	Erkurt et al. (2007)
Laccase	Biodegradation of Remazol Brilliant Blue R, Remazol Brilliant Blue RR, Remazol Red RR, Remazol Yellow RR	*Trametes versicolor*	Christian et al. (2005)

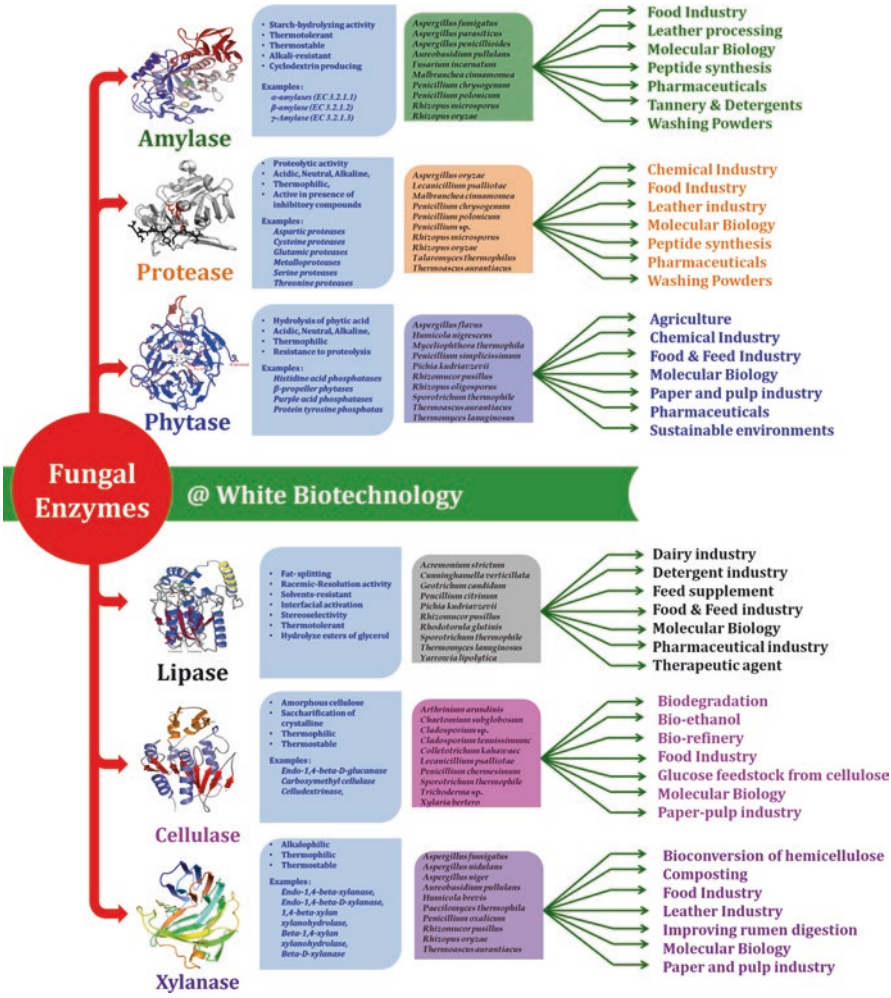

Fig. 1.3 Fungal enzymes and their biotechnological applications in diverse sectors

endohydrolysis of 1,4-β-D-xylosidic linkages in xylan and arabinoxylan. These have been reported from diverse groups archaea, bacteria, as well as fungi (Verma and Satyanarayana 2012).

1.3.1.1 Xylanases

Xylanases find wide application in the baking industry. They are used to improve the strength of the gluten network, ultimately improving the quality of bread (Butt et al. 2008; Gray and Bemiller 2003). They make the dough more tolerant to different qualities of flour and also to variations that occur in processing parameters.

Xylanases are known to transform water-insoluble hemicelluloses into soluble form, which binds water in the dough, thus reducing the firmness of the dough and further improving firmness of dough as well as creation of finer and more uniform crumbs. Finally, the dough becomes more machine friendly and does not stick to different parts of the machinery (Rouau 1993). Adding more, xylanases also decrease the sheeting work requirements and considerably increase the volume of the baked bread (Dervilly et al. 2002; Harbak and Thygesen 2002). Furthermore, the addition of xylanases during the processing of dough is expected to enhance the concentration of arabinoxylo-oligosaccharides in bread, which is beneficial for human health (Bhat 2000). Thus, xylanases greatly improve the quality of biscuits, cakes, and other baked products (Poutanen 1997).

1.3.1.2 Phytases

Phytases are a class of phosphatases which exhibit capability to release at least one phosphate from phytate in vitro. Microbial phytases are among the most promising for biotechnological applications. Extracellular phytate-degrading enzymes have been reported in the molds and yeast. Phytases have been reported from *Aspergillus*, *Candida*, *Humicola*, *Fusarium*, *Myceliophthora*, *Penicillium*, *Pichia*, *Rhizomucor*, *Rhizopus*, *Sporotrichum*, *Schizosaccharomyces*, *Thermoascus*, *Williopsis*, *Yarrowia*, and *Zygosaccharomyces* (Gupta et al. 2014; Kaur et al. 2017; Kumar et al. 2016, 2017; Mitchell et al. 1997; Nampoothiri et al. 2004; Pable et al. 2014; Ushasree et al. 2017; Yadav et al. 2017a) (Fig. 1.4). Phytase has been known to be an outstanding bread-making improver (Afinah et al. 2010). It is known that adding commercial fungal phytase from *Aspergillus niger* in the dough ingredients consisting of fiber formulation speeds up proofing, greatly improves bread shape, increases specific volume, and adds to softness to the crumb. All these improvements in bread quality have been demonstrated to be associated with an indirect impact of phytase on α-amylase activity (Afinah et al. 2010; Greiner and Carlsson 2006).

1.3.1.3 Lipases

Lipases or triacylglycerol acylhydrolases are ubiquitous enzymes found in animals, plants, fungi and bacteria. They are of physiological significance and also possess industrial potential. Lipases exhibit the sole feature of acting at the interface between an aqueous and a non-aqueous phase. They are able to synthesize esters from glycerol and long chain fatty acids when the water activity is low (Aravindan et al. 2007). The use of lipases in the baking industry is in fact recent when compared to α-amylases and proteases. Extracellular secretion of lipases has been well known in fungi, mainly in hyphomycetes, zygomycetes (Akhtar et al. 1983; Gopinath et al. 2013). Lipolytic activity has been reported in *Aspergillus* sp., *Acremonium strictum*, *Candida antarctica*, *Candida rugosa*, *Cunninghamella verticillata*, *Humicola lanuginose*, *Kluyveromyces* sp., *Lachancea* sp., *Lipomyces starkeyi*, *Mucor* sp.,

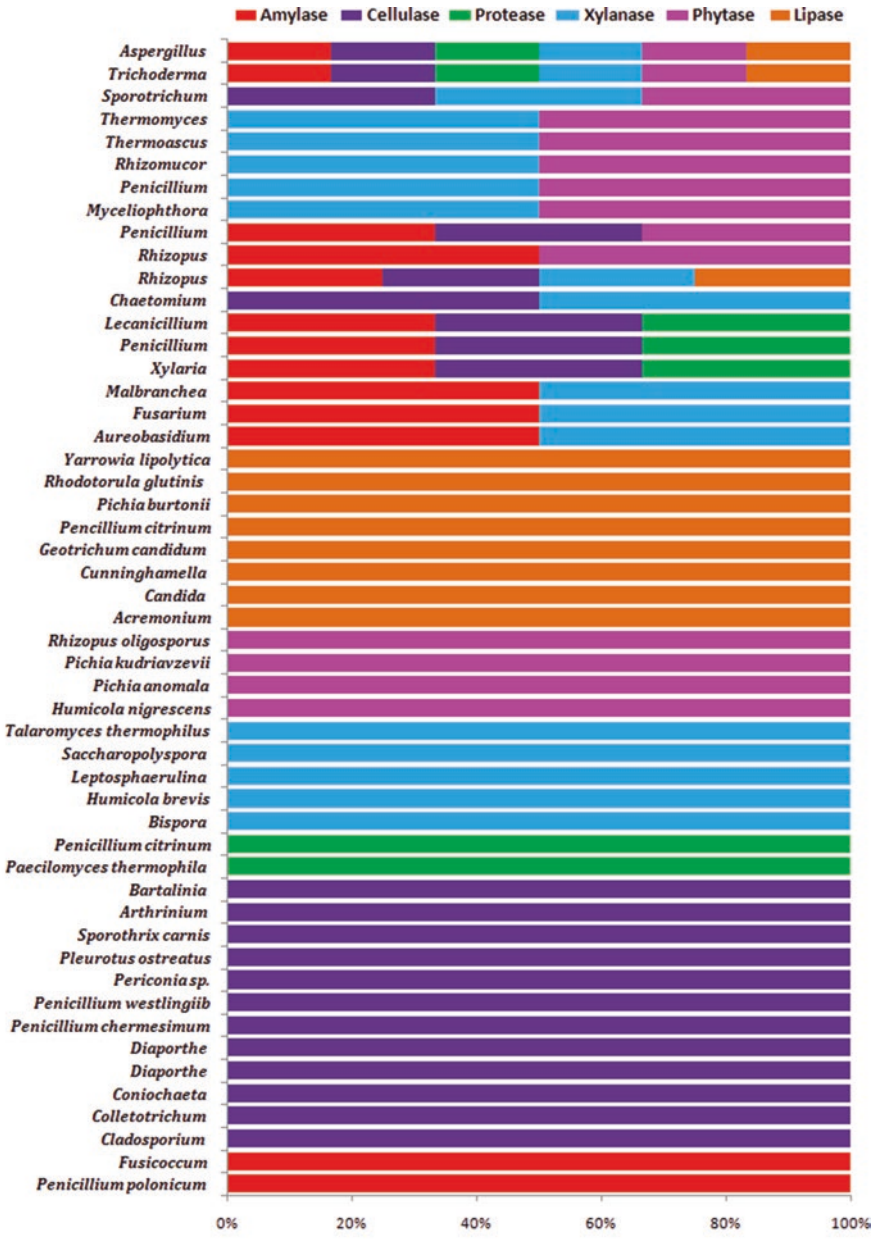

Fig. 1.4 Industrial enzymes producing of fungal community

Penicillium sp., *Pichia burtonii*, *Rhizopus* sp., *Rhodotorula glutinis*, *Saccharomyces lipolytica*, *Torulaspora* sp., *Trichoderma lentiforme*, *Yarrowia lipolytica*, and *Zygosaccharomyces* sp. (Romo-Sánchez et al. 2010; Verma et al. 2017c; Wang et al. 2018). Lipases are known to enhance the flavor content of bakery products (Ray 2012). They have also been found to be effectual in reducing the initial firmness and enhancing the specific volume of breads (Keskin et al. 2004). Texture and softness could be improved by lipase catalyzation (Laboret and Perraud 1999; Ray 2012).

1.3.1.4 Laccases

Laccase is a kind of copper-containing polyphenol oxidase oxidizing diamines, polyphenols, methoxy-substituted phenols, and a significant range of other compounds. These can be obtained from bacteria, fungi, and plants (Gianfreda et al. 1999). Most biotechnologically useful laccase is of fungal origin. There are more than 60 fungal strains from *Ascomycota*, *Basidiomycota*, *Zygomycota* that are reported to have maximum laccase activity (Arpita and Kumar 2018; Kiiskinen et al. 2004b). Laccases are utilized in the baking industry especially in the process of bread making where its activity results in improved volume, texture, flavor, and freshness of bread. The use of laccases also improves the machinability of the dough (Minussi et al. 2002). Furthermore, it also improves the crumb structure and softness of the baked product as well as increases the strength and stability and also reduces the stickiness.

1.3.1.5 Amylases

Amylases are one of the most important and oldest used industrial enzymes. Amylases hydrolyze starch molecules into diverse products such as dextrins and progressively smaller polymers composed of glucose units (Gupta et al. 2003a; Windish and Mhatre 1965). These enzymes are of great importance in present-day biotechnology possessing different applications such as in food, fermentation, textile, and paper industries (Gupta et al. 2003a; Yadav et al. 2016b). Amylases can be obtained from numerous sources, such as plants, animals, and microorganisms, but microbial sources are the preferred ones which are able to meet industrial demands. In fact, fungal α-amylases have been permitted as bread additives since 1955 in the USA and in 1963 in the UK after confirmation of their Generally Recognized As Safe (GRAS) status (Pritchard 1992). Fungal sources are limited to terrestrial isolates, mostly to *Aspergillus* and *Penicillium* (Kathiresan and Manivannan 2006). *Aspergillus oryzae* and *Aspergillus niger* produce significant quantities of enzymes that are used expansively in the industry. The thermophilic fungus *Thermomyces lanuginosus* is an excellent producer of amylase (de Souza 2010).

The baking industry has been utilizing this enzyme for hundreds of years for manufacturing a wide array of high-quality products. The addition of α-amylases to

the dough enhances the fermentation rate and reduces of the viscosity of dough, resulting in improvements in the volume and texture of the product (de Souza 2010). Furthermore, it is also known to generate extra sugar in the dough, which greatly improves the crust color, taste, and toasting qualities of the bread. Adding more, amylases also possess an anti-staling effect in bread baking, and they improve the softness retention of baked goods, further causing an increase in the shelf life of these products (Gupta et al. 2003a; Van Der Maarel et al. 2002).

1.3.2 Pharmaceutically Important Fungal Enzymes

1.3.2.1 Tannases

Tannases comprise two classes of enzymes, including the tannin acyl hydrolases and ellagitannin acyl hydrolases. Microbes are the most preferred source of tannases. Fungi including *Aspergillus* sp., *Paecilomyces variotii*, *Penicillium* sp., *Verticillium* sp., (Battestin and Macedo 2007; González et al. 2017; Kasieczka-Burnecka et al. 2007) have been known to produce tannases. Tannases are known to produce gallic acid and propyl gallate (Belmares et al. 2004; Kar et al. 2002). The former finds it use in the pharmaceutical industry for the synthesis of antibacterial drugs (Belmares et al. 2004).

1.3.2.2 Lipases

Microbial lipases are used to enrich polyunsaturated fatty acids (PUFAs) from animal and plant lipids, such as borage oil, menhaden oil, and tuna oil (Dong et al. 1999). A lot of polyunsaturated fatty acids are necessary for normal synthesis of lipid membranes and prostaglandins. Free polyunsaturated fatty acids and their mono- and diacylglycerides are used for the production of an array of pharmaceuticals (Sharma et al. 2001; Yadav 2015). Additionally, the lipases possess the capability to resolve racemic mixtures by the synthesis of a single enantiomer which is currently exploited for drug production by the pharmaceutical industry (Houde et al. 2004). Furthermore, the lipases are also utilized for the synthesis of a range of enantiopure molecules, for instance, esters, carboxylic acids, amides, and alcohols. These molecules are used in anti-inflammatory drugs such as ibuprofen and naproxen (Akimoto et al. 1999); anticancer drugs (Taxol®, spergualin); antiviral drug, for instance, lobucavir; antihypertensive drug including captopril; anticholesterol drugs such as squalene synthase inhibitor; and anti-Alzheimer disease drug, i.e., [*S*]-2-pentanol and vitamin A (Kovac et al. 1996). Lipases also have the capacity to catalyze synthetic reactions which has led to the production of lifesaving drugs. There are also reports on use of immobilized lipases for the synthesis of nutraceuticals (Aravindan et al. 2007).

1.3.3 Fungal Enzymes in Dairy Industry

Dairy industry is another important industry in which enzymes form an important segment. There are different enzymes that are used in food industry for development and enhancement aroma, color as well as flavor and higher yield of milk products.

1.3.3.1 Proteases

Proteolytic enzymes are also referred to as peptidases, proteases, and proteinases. They possess the capability to hydrolyze peptide bonds in the molecules of the proteins. Proteases have been categorized into endopeptidases and exopeptidases (Singh and Kumar 2019) and have been obtained from different groups of the organisms including animals, plants, bacteria, and fungi. But at industrial level, either the bacterial or the fungal proteases are utilized. Microbes are known to secrete intracellular as well as extracellular proteases under solid state as well as sub merged fermentation process. *Aspergillus flavus*, *Aspergillus fumigatus*, *Aspergillus niger*, and *Aspergillus oryzae* are known to be the best sources of protease enzyme (Singh and Kumar 2019). Proteases are used for the acceleration of cheese ripening and for the modification of milk proteins for reducing the allergenic properties of cow milk products for infants (Qureshi et al. 2015).

1.3.3.2 Lipases

The lipases are used for the hydrolysis of milk fats, pronounced cheese flavor, low bitterness, and prevention of rancidity. Lipases in combination with many other enzymes such as protease or peptidases are used to create good cheese flavor with low levels of bitterness (Wilkinson 1995). These are also used for the lipolysis of butter, fat, and cream (Aravindan et al. 2007). Lipases are also added to Italian cheese, such as Romano, Parmesan, and provolone, to enhance their flavor (Custry et al. 1987).

1.3.3.3 Lactase

Lactase or β-galactosidase catalyzes the hydrolysis of lactose into glucose and galactose. Lactases can be obtained from animals, plants, bacteria, fungus, yeasts, and molds. Commercial production of lactase enzymes is developed from *Aspergillus niger*, *Aspergillus oryzae*, and *Kluyveromyces lactis* (Mehaia and Cheryan 1987). These are used as a digestive aid and to increase the solubility as well as sweetness in milk products (Qureshi et al. 2015; Soares et al. 2012). It is also utilized either to minimize or remove lactose content of milk for lactose-intolerant people so that diarrhea, severe tissue dehydration, and sometimes fatal consequences could be

prevented (Kardel et al. 1995; Mahoney 1997; Pivarnik et al. 1995). Lactases are also used to enhance the lactase-treated milk. Furthermore, these also assist in manufacturing of ice cream as well as preparation of yogurt (Singh and Kumar 2019).

1.3.3.4 Catalase

Another important enzyme for dairy industry includes catalase. These break down hydrogen peroxide into water and oxygen molecules, thus protecting cells from oxidative damage by reactive oxygen species (Singh and Kumar 2019). Commercial catalases are produced from *Aspergillus niger* through a solid-state fermentation process (Fiedurek and Gromada 2000). Catalases have a special application in cheese production. For the production of some cheeses such as Swiss cheese, hydrogen peroxide which is a strong oxidizer and toxic to the cells is used in the state of pasteurization. It is used to retain natural enzymes of milk that are useful for the finished product and flavor development of the cheese (Abada 2019; Perin et al. 2017).

1.3.3.5 Rennet

Rennet is one of the famous exogenous enzymes which is used in dairy processing, and has been used since 6000 BCE (Abada 2019). It is a combination of chymosin and pepsin and is used for coagulation of milk into solid curds for the production of cheese and liquid whey (Singh et al. 2016a). Rennin acts on the milk protein in two stages, by enzymatic and nonenzymatic action, finally resulting in coagulation of milk. Many microorganisms are known to produce rennet-like proteinases which can substitute the calf rennet. But *Aspergillus oryzae*, *Endothia parasitica*, *Irpex lactis*, *Rhizomucor pusillus*, and *Rhizomucor miehei* are expansively used for rennet production in cheese manufacture (Qureshi et al. 2015).

1.3.4 Fungal Enzymes in Textile Industry

Textile industry is another important industry utilizing diverse enzymes. In fact, textile industry is one of the sectors of industry holding major share in the global pollution. Thus, enzymes play a chief role in processing of the textiles and have become central part of the textile industry. The use of the enzymes in manufacturing of textile has been a tradition. Amylases are used for desizing process that involves the removal of starch from the fabric. Thus, α-amylase cleaves starch particles randomly into water-soluble components which are removed by washing (Mojsov et al. 2018). The α-amylases selectively remove the size and do not attack the fibers (Ahlawat et al. 2009; Gupta et al. 2003a; de Souza 2010).

Cellulases act on cotton yarns facilitating the removal of the indigo dye from the surface of the yarn. Sizing agents such as starch are applied to yarn before fabric production to ensure a fast and secure weaving process (Doshi and Shelke 2001). The stones initially used in textile industry have been replaced by cellulases which prevent the damage to the garments as well as the washing machine. Furthermore, it also removes the need of rinsing garments again and again to eliminate dust and thus reduces effluent load (Paul and Naik 1997).

Lipases are used to aid in the removal of size lubricants so that the fabric could be provided with better absorbency for enhanced levelness in dyeing. It also reduces the frequency of cracks and streaks in the denim abrasion systems. Commercial preparations that are used for the desizing of denim and other cotton fabrics have lipase enzymes (Handelsman et al. 1998; Hasan et al. 2006).

Pectinases in combination with amylases, cellulases, lipases, and other hemicellulolytic enzymes for the removal of sizing agents have reduced the use of harsh chemicals in the textile industry, ultimately resulting in a lower discharge of waste chemicals to the environment, thereby improving both the safety of working conditions for textile workers and the quality of the fabric (Hoondal et al. 2002).

Proteases are used for removal of the dull and stiff gum layer of sericin from the raw silk so that softness as well as the lusture could be achieved. Adding more, the treatments with proteases also modify the surface of wool and silk fibers to provide unique finishes (Doshi and Shelke 2001).

1.3.5 Fungal Enzymes in Food and Feed Industry

During the past four decades, the rapid development of enzyme industry is due to the advancement in biotechnology. As during ancient time, enzymes were used for the production of food products such as cheese, beer, wine, and vinegar (Kirk et al. 2002). The plants, fungi, bacteria, and yeasts are known for their ability to produce most enzymes. The syntheses of enzymes by microbes are more advantageous as compared to enzymes synthesized by animal or vegetable sources. The microbial enzymes are appropriate biocatalysts for various industrial applications as they comprise lesser production costs, large-scale production in industrial fermenters, opportunity of genetic manipulation, and fast growth of culture (Hasan et al. 2006). Scientists have high intentional interest in the discovery of new microbial sources having higher effectiveness to synthesize enzymes and that are nontoxic to humans. In numerous industries fungi acquired immense significance. *Aspergillus* isolated from soil, decomposing plants, and air reported to produce a great number of extracellular enzymes many of which are applied in biotechnology. There is a remarkable interest in *Aspergillus niger,* as it is broadly useful in modern biotechnology (Frisvad et al. 2008) and is classified as GRAS (Generally Recognized as Safe) by the Food and Drug Administration (FDA) (Couto and Sanromán 2006). One of the enzyme, pectinases reported to be isolated from *Aspergillus*, *Rhizopus*, *Trichoderma*, *Pseudomonas*, *Penicillium*, and *Fusarium* (De Gregorio et al. 2002) is used in the

food industries for ripening of fruit, wine industries, tomato pulp removal out improvement of protein in baby food, extraction of oil and animal nutrition. Table 1.2 represents fungal enzymes associated with food and feed bioprocessing.

1.3.6 Fungal Enzymes in Leather Industry

Leather is utilized in the manufacture of a huge number of commercial commodities, and it has gained a status symbol as one of the highest foreign trade earners and belongs to the elite of society. One of the most important industrial enzymes is protease. Filamentous fungi are known for their ability to synthesize proteolytic enzymes (Attawut et al. 1981). Many researchers have reported *Aspergillus* species produces extracellular alkaline proteases (Anandan et al. 2007). Alkaline proteases are prominently playing their role in unhairing and bating processes in leather industry. In an experiment, Chellapandi (2010) reported *Aspergillus flavus* and *Aspergillus terreus* as the probable strains for the production of tannery protease in submerged fermentation and the protease was used for unhairing processes at lab scale in tannery. In wide alkaline conditions at 50 °C, the protease showed the good activity signifying the opportunity by which it can be used in leather and detergent industry. Fungal expression systems are capable of producing larger quantities of enzymes than bacterial expression systems. Filamentous fungi, for instance, *Aspergillus*, have been the organism of preference for large-scale production of bulk industrial enzymes, as the fungi can be grown on moderately inexpensive (agricultural waste) media and the fungi can secrete bulk quantities of enzymes (Bergquist et al. 2002).

In a study by Anandan et al. (2007), *Aspergillus tamari* produces extracellular alkaline protease and the enzyme is useful in removing hair from cattle hide. The alkaline protease was homologous to the alkaline protease expressed by *Aspergillus viridinutans*. In leather-making process in a tannery, a raw hide is subjected to a sequence of chemical treatments before tanning and finally converted to finished leather. In these treatments, alkaline proteases may play a vital role by replacing these hazardous chemicals especially involved in soaking, dehairing, and bating. Enlarged practice of enzymes for dehairing and bating is effective in saving time with better quality leather and also prevent pollution (Zambare et al. 2011). Besides, studies have confirmed alkaline protease secreted by *Conidiobolus coronatus* has been evaluated broadly in tanneries and finds relevance in pre-tanning operations in leather manufacture (Laxman et al. 2005). The production of alkaline protease by an *Aspergillus flavus* strain was used as a depilation agent was confirmed by experiments in a tannery. The enzyme exhibited maximum activity at both pH 7.5 and pH 9.5 (Malathi and Chakraborty 1991).

Table 1.2 Fungal enzymes associated with food and feed bioprocessing

Industries	Enzymes	References
Fruit extraction	Amylase, amyloglucosidase, cellulase, pectinase, pentosanase, limonoate, dehydrogenase, naringinase	Albersheim (1966)
Flavors	Glucanase, peptidase, proteinase, esterase, lipases, amylase	Shahani et al. (1976)
Animal oil/fats	Esterases, lipases, proteinase	Beldman et al. (1984)
Fats	Esterase, glucose oxidase, lipases	Bobek et al. (1994)
Starch	Amylase, amyloglucosidase, cellulase, glucanase, hemicellulase, isomerase, lipase, phospholipase, pectinases, proteases	Okolo et al. (1995)
Fruit extracts	Anthocyanase	Albersheim (1966)
Botanical extraction	Amylase, amyloglucosidase, cellulase, glucanase, hemicellulase, pectinases, proteases	Alkorta et al. (1998)
Confectionery	Amylase, invertase, pectinase, proteinase	Stroh (1998)
Dairy products	Lactase, proteinase, sulfhydryl oxidase, lactoperoxidase, lysozyme, peroxidase, catalase	Alkorta et al. (1998)
Debittering	Peptidase, naringinase	Alkorta et al. (1998)
Dairy products	Lactase, proteinase, sulfhydryl oxidase, lactoperoxidase, lysozyme, peroxidase, catalase	Beauchemin et al. (1999)
Cheese	Rennet, lipase, proteinases	Freitas and Malcata (2000)
Dairy products	Lactase, proteinase, sulfhydryl oxidase, lactoperoxidase, lysozyme, peroxidase, catalase	Archer (2000)
Biscuits	Amylases, cellulases, hemicellulases, proteinases, pentosanases	Taniwaki et al. (2001)
Breads	Amylases, amyloglucosidases, cellulases, glucanases, glucose oxidase, hemicellulases, lipases, pentosanases, proteinases	Taniwaki et al. (2001)
Brewing	Acetolactase, decarboxylase, amylases, amyloglucosidase, cellulase, glucanase, lipase, pentosanase, proteinase, xylanase	Okamura et al. (2001)
Fish	Proteinase	Prasad (2001)
Fruit extraction	Amylase, amyloglucosidase, cellulase, pectinase, pentosanase, limonoate, dehydrogenase, naringinase	Kashyap et al. (2001)
Wine	Amylase, amyloglucosidase, cellulase, glucanase, hemicellulase, pectinases, proteases, glucose, oxidase, catalase, pentosanase, anthocyanase	Okamura et al. (2001)
Alcohol	Amylase, amyloglucosidase, b-glucanases, cellulases, cellobiase, pectinase, proteinases	Sharma et al. (2002)
Butter and butter oils	Catalase, glucose oxidase, lipase	Gupta et al. (2003b)

(continued)

Table 1.2 (continued)

Industries	Enzymes	References
Fructose	Glucose isomerase, inulinase, amylase, amyloglucosidase, cellulase, glucanases, hemicellulases, isomerase, lipase, phospholipase, pectinases, proteases	Sørensen et al. (2004)
Fruit, cloudy juices	Amylases pectinases, cellulases, proteinase	Mantovani et al. (2005)
Fruit pulps	Pectinase, amylase, amyloglucosidase, cellulase, glucanase, hemicellulase, pectinase, protease	Mantovani et al. (2005)
Tea	Cellulase, glucanase, pectinase, tannase	Pasha and Reddy (2005)
Vegetable and fruit processing	Cellulases, macerating enzymes, pectinases	Mantovani et al. (2005)
Animal feed	Amylase, glucoamylases, glucanase, cellulases, pentosanases, xylanases, proteinases, phytases xyloglucanases, galactomannanases, arabinofuranosidases, ferulic acid esterases	Wang et al. (2006)
Fruit juice	Amylase, amyloglucosidase, cellulase	Semenova et al. (2006)
Protein	Amylase, cellulase, glucanase, hemicellulase, pectinase, protease	Semenova et al. (2006)
Egg processing	Proteinase, lipase phospholipase, catalase, glucose oxidase	Singh et al. (2007)
Coffee	Cellulase, hemicellulases, galactomannanase, pectinase	Soccol et al. (2008)
Flavors	Glucanase, peptidase, proteinase, esterase, lipases, amylase	de Souza (2010)
Starch	Amylase, amyloglucosidase, cellulase, glucanase, hemicellulase, isomerase, lipase, phospholipase, pectinases, proteases	de Souza (2010)
Starch	Amylase, amyloglucosidase, cellulase, glucanase, hemicellulase, isomerase, lipase, phospholipase, pectinases, proteases	Rana et al. (2013)
Fruit pulps	Pectinase, amylase, amyloglucosidase, cellulase, glucanase, hemicellulase, pectinase, protease	Jalis et al. (2014)
Wine	Amylase, amyloglucosidase, cellulase, glucanase, hemicellulase, pectinases, proteases, glucose, oxidase, catalase, pentosanase, anthocyanase	Garg et al. (2016)
Fruit extraction	Amylase, amyloglucosidase, cellulase, pectinase, pentosanase, limonoate, dehydrogenase, naringinase	John (2017)
Animal feed	Amylase, glucoamylases, glucanase, cellulases, pentosanases, xylanases, proteinases, phytases xyloglucanases, galactomannanases, arabinofuranosidases, ferulic acid esterases	Singh and Yadav (2018)
Fruit juice	Amylase, amyloglucosidase, cellulase	Zhang et al. (2018)

1.3.7 Fungal Enzymes in Beverages Industry

The beverages industry is also known as the drink industry and is one of the largest food processing industries which manufactures carbonated beverages and alcoholic drinks. The beverage industry is categorized into two major groups and eight subgroups. Alcoholic beverage categories include distilled spirits, wine, and brewing whereas nonalcoholic group consist of soft drink syrup manufacture, fruit juices, the coffee industry and the tea industry (*Encyclopaedia of Occupational Health and Safety*). In the production of beer and other types of malted liquor, such as whiskey, enzymes play a crucial role. Enzymes are also useful in production of wine, serving in the safeguarding of wine quality, sometimes over many years in storage. Enzymes also endorse clarification, filtration, and stabilization and lessen time of fermentation (Kashyap et al. 2001). Due to rising consciousness about health among the people, the requirement of fruit juices is rising gradually. The enzymes used are mainly pectinase, cellulase, hemicellulase, etc. for extraction of juices. Enzymes also stop darkening of juices.

The enzymes naringinases are used in orange and grapefruit processing to improve pulp washing, to increase the recovery yield of essential oils, and to debitter and clarify the juice (Godfrey and West 1996), and the most bitter compounds present in citrus juices are naringin, limonin, and neohesperidin (Marwaha et al. 1994). The existence of bitterness has been a chief drawback in the profitable approval of juice. Naringinase has important applications in debittering of fruit juice. *Coniothyrium diplodiella* one of the phytopathogenic organisms reported to produce a pectic enzyme that had naringinase activity and have a high potency (Takiguchi 1962). In another study, Fukumoto and Okada (1974) reported production of naringinase enzyme using *Penicillium* sp. According to study, enzymes assimilate pectin, starch, proteins, and cellulose of fruits and vegetables and assist enhancement in yields and decrease in processing time (Mojsov 2012).

The enzyme acidic pectinases used in the fruit juice industries and wine making often come from fungal sources, especially from *Aspergillus niger*. Alkorta et al. (1998) reported pectinases and cellulases give a juice yield up to 100%. Rhamnogalacturonase, a type of pectinase, was found initially in *Aspergillus aculeatus* but later was also found in other species of *Aspergillus* (Schols et al. 1990). Pectic enzymes isolated from fungi, usually *Aspergillus niger*, *Penicillium notatum*, or *Botrytis cinerea*, are helpful in wine making (Fogarty and Kelly 2012; Robertson 1977). Pectinolytic enzymes are involved in the retting and degumming of jute, flax, hemp, ramie, kenaff (*Hibiscus sativa*), and coir from coconut husks (Brühlmann et al. 1994; Chesson 1980). Retting is a fermentation process in which certain fungi (e.g., *Aspergillus*, *Penicillium*) decompose the pectin of the bark and release fiber (Sharma and Robinson 1983).

1.3.8 Fungal Enzymes in Detergent Industry

Due to continuous biotechnological research, in certain niches the detergent market continues to make improvement. Not only it is significant that washing garments will be in pristine form, but ecological factors are also a major concern. Biotechnology can considerably add to production of detergents still safer for the environment. In the detergent industry, the usage of protease enzyme has long history. The first method of cleaning the fabrics contained the enzymes but was not that much effective. Today, the enzyme proteases are found in most of detergents. At high pH, the enzyme possesses the stability (Salleh et al. 2006). Detergents are used for dishwashing, laundering, and domestic, industrial, and institutional cleaning (Schäfer et al. 2005). The enzyme removes the protein, starch, oil, and fats in the form of stain (Hasan et al. 2010). Sometimes a blend of enzymes, together with proteases, amylases, pectinases, cellulases, and lipases, are used to amplify effectiveness on cleanup of stain (Li et al. 2012). The second type of enzymes used in the detergent formulation is amylases; about 90% of all liquid detergents contain these enzymes (Payen and Persoz 1833). The enzyme lipases represent one of the most important groups of biocatalysts and have been isolated from many species of plants, animals, bacteria, fungi, and yeasts. Lipases are used in household dishwashers and industrial laundry where they function in the removal of fatty residues (Kumar et al. 1998; Vulfson 1994). Jaeger et al. (1994) in their study reported marketable detergent formulations synthesized from *Humicola lanuginose*; later the gene was cloned in *Aspergillus oryzae* for increasing the yield. The enzyme lipase was isolated from *Aspergillus oryzae* and *Trichosporon asahii MSR 54* as the enzyme performs its action of removing oil stains at ambient temperature (Gerhartz 1990; Kumar et al. 2009).

The enzymes have beneficial effect on the ecosystem as they provide environment profit by dropping energy utilization through shorter washing times, lower washing temperatures, and compact water consumption as well as they also contain less bleaching agents. The production processes of enzymes are by fermentation technologies that employ renewable resources (Olsen and Falholt 1998). Germano et al. (2003) in his study reported protease enzyme produced by a wild strain of *Penicillium* sp. The crude enzyme was well-suited with marketable detergents, retaining their 50–60% activities. The enzyme also offered fine constancy toward oxidizing agent. Devi et al. (2008) reported *Aspergillus niger* produces alkaline protease. At 40 °C the enzyme retained more than 50% activity after 60 min incubation in the presence of detergents such as Tide, Surf, Wheel, and Henko signifying its appropriateness for use in detergent industry. Savitha et al. (2011) investigated the effectiveness of proteases enzyme isolated from *Graphium putredinis* and *Trichoderma harzianum* fungal strain. The result indicated proteases from these fungal strains can be used as potential additives in the commercial detergents and showed a good washing performance. The use of fungal protease minimizes the toxicity of harsh chemicals engaged in laundry detergents and could offer a safer environ in the pollution encumbered world.

1.3.9 Fungal Enzymes in Organic Synthesis Industry

The organic synthesis industry belongs mainly to the chief branches of the modern chemical industry. During last century mostly organic compounds were obtained from raw plant and animal materials. In the recent time, foremost resources for the synthesis of diverse organic compounds are natural gases, oil-refinery gases, coke gases, crude oil, etc. The obtained ability of different organic compounds by means of synthetic method may completely replace the natural sources. Enzymes are gaining popularity even for the production of fine chemicals due to additional cost-effective and purity of products in a tolerable manner which are also eco-friendly (Nagasawa and Yamada 1995). Over traditional methods enzymes are favored in organic synthesis industry for their several benefits such as high catalytic effectiveness, high selectivity, eco-friendly, and easier separation (Johannes et al. 2006; Schmid et al. 2001). One of the most important enzymes used in organic synthesis is the lipases which resulted in the formation of alcohols, acids, esters, (S, R)-2, 3-pethoxyphenylglycyclic acid, etc. (Gentile et al. 1992; Hasan et al. 2006; Jaeger and Reetz 1998). The enzyme lyases are involved in organic synthesis of cyanohydrins from ketones, acrylamide from acrylonitrile, and malic acid from fumaric acid (Faber 1992; Zaks 2001).

The enzyme laccases are multi-copper-containing oxidases found in plants, insects, and bacteria (Claus 2003; Dittmer et al. 2004; Kramer et al. 2001). There are over 60 fungal strains belonging to *Ascomycetes*, *Deuteromycetes*, and particularly *Basidiomycetes* which are reported to show laccase activities mostly of biotechnological application (Baldrian 2006). The industrial applicability of laccase includes enzymatic modification of fibers and dye bleaching in the textile and dye industries; detoxification of lignocellulose hydrolysates for ethanol production; and construction of biosensors and biofuel cells (Abadulla et al. 2000; Kunamneni et al. 2008). In organic synthesis, laccases have been employed for the oxidation of functional groups, the coupling of phenols and steroids, and the construction of carbon nitrogen bonds and in the synthesis of complex natural products and more (Baiocco et al. 2003; Barilli et al. 2004; d'Acunzo et al. 2002; Nicotra et al. 2004). The heterologous expression of active laccases has been reported mainly in filamentous fungi *Aspergillus oryzae*, *Aspergillus niger*, *Aspergillus sojae*, and *Trichoderma reesei* (Hatamoto et al. 1999; Kiiskinen et al. 2004a; Téllez-Jurado et al. 2006).

1.4 Fungi and Fungal Enzyme for Sustainable Environments

In the biodegradation of organic compounds in wastewater, microbes play the significant role. In the food industries, filamentous fungi are often cultivated (Barbesgaard et al. 1992). One of the best alternatives for the treatment of high-strength wastewater is the usage of filamentous fungi. The treatment of wastewater

with fungi not only converts organics into high-value fungal protein but also synthesizes dewaterable fungal biomass, which further can be used for animal feed and also in human diets (Guest and Smith 2002; Zheng et al. 2005). Enzymes secreted by fungi are more efficient in metabolizing complex carbohydrates, for instance, starch (Jin et al. 1998; Van Leeuwen et al. 2003). The fungi also include a group of extracellular enzymes which assist in the biodegradation of recalcitrant compounds such as phenolic compounds, dyes, and polyaromatic hydrocarbons (PAH) (D'Annibale et al. 2004; Jaouani et al. 2005). The treatment of waste streams containing hazardous or xenobiotic organic pollutants can be achieved by enzyme-mediated activity synthesized by fungi. During all phases of the fungal life cycle, the enzymes are secreted (Ryan et al. 2005). Secretion of both specific and nonspecific extracellular enzymes by fungi laid the interest of researchers toward its study.

White-rot fungi were reported to secrete certain enzymes, for example, ligninase, phenol-oxidase, and manganese-peroxidase, that are proficient in degrading lignin, phenol, dyes, and various other xenobiotic pollutants (Esposito et al. 1991; Libra et al. 2003). A little information compiled by the National Collection of Industrial Microorganisms (NCIM), National Chemical Laboratory, New Delhi, from WFCC-MIRCEN World Data Centre for Microorganisms (WDCM) record for fungal species degrading specific types of wastes such as *Humicola grisea* that degrades raffinose, *Alternaria tenuis*, *A. niger*, and *Trichoderma viride* that degrade plastic; *P. chrysosporium* that degrades lignin, *Myrothecium verrucaria* and *Trametes hirsuta* that degrade cellulose-rich waste. During the duration of treatment of wastewater, cell walls of fungi participate in biosorption of toxic compounds (Kapoor and Viraraghavan 1995). The numbers of enzymes isolated from fungus are concerned with the degradation of toxic pollutants. A number of enzymes such as amidases, amylases, amyloglucosidases, cellulases, glucoamylases, lipases, pectinases, and proteases are engaged for the treatment of waste (Margesin et al. 1999; Riffaldi et al. 2006; Karigar and Rao 2011).

1.5 Fungal Communities in Food and Feed Processing

The fungi as food and feed are very nutritive as they are good source of essential and nonessential amino acids. Since ancient times, fungi have been used as source of food by humans (Dupont et al. 2016; Silar 2013). The most common being the edible sexual structures of basidiomycetes and ascomycetes, so-called mushrooms, which are produced mostly in wood and represent a rich source of proteins, with low fat content. Fungi are ideal food because they have a quite high protein content typically 20–30% dry matter as crude protein. Fungal biomass is also a good source of dietary fiber and is almost free of cholesterol and easy to digest. The chitinous wall of fungi provides a source of dietary fiber, further also containing B vitamins, are typically low in fat. Thus, fungal protein foods effectively compete with animal

protein foods on health grounds (Moore and Chiu 2001). A small number of plant-pathogenic fungi are also eaten, for instance, *Ustilago maydis*, corn smut fungus which produces black tumors on maize. These tumors are considered a delicacy in Mexico, where it is called huitlacoche (Valverde et al. 2012).

1.5.1 Use of Fungi in Dietary Food

The consumption of fungal food is increasing day by day on a global basis with rise in public concern about dietary and health issues. Especially, for the vegetarians either freshly cooked mushrooms or processed foods, beverages and dietary supplements of fungal origin are good alternative (Ghorai et al. 2009). Mushrooms have been used by people since Neolithic times for food as well as for medicinal purposes (Dugan 2008), but the best-known use of mushrooms in the Western world is as a food material. At present, there are at least 1100 species of mushrooms eaten in more than 80 countries (Baars 2017). Most of the highly appreciated mushrooms are mycorrhizal species which live in a symbiotic relation with trees. Mushrooms contain significant amounts of copper, iron, potassium, and phosphorus. There are different species of mushrooms which are cultivated in different parts of the world such as *Pleurotus* species, i.e., oyster mushroom. Other includes *Lentinula edodes* (shiitake), *Flammulina edodes* (enokitake), *Volvariella volvacea* (straw mushroom), *Auricularia* sp. (wood ear mushroom), and *Agaricus bisporus* (button mushroom). Then, there are porcini mushrooms (*Boletus edulis* group) offered mostly in a dried form to consumers and truffles (*Tuber* species) that are collected in nature (Baars 2017). Morel mushrooms (*Morchella* sp.) include a variety of species which are known to grow in temperate zones throughout the world. Black morels such as *Morchella angusticeps*, *Morchella conica*, *Morchella costata*, and yellow morels including *Morchella deliciosa* and *Morchella esculenta* are the most popular edible species.

1.5.1.1 *Lentinula edodes* (Shiitake)

It is known to be rich in proteins with all essential amino acids. Further, it is a rich source of vitamin B and also is known to contain moderate levels of minerals. Shiitakes produce vitamin D2 when their internal ergosterol is exposed to ultraviolet B rays from sunlight or broadband UVB fluorescent tubes (Ko et al. 2008). It is known to contain adenine and choline which are effectual in preventing the occurrence of cirrhosis of the liver as well as vascular sclerosis; it further also possesses medicinal properties as it contains tyrosinase which lowers blood pressure. Furthermore, it also consists of an active polysaccharide, lentinan which is known to reduce cancer as well as cholesterol and causes enhancement of TH1 response (Ghorai et al. 2009; Murata et al. 2002; Rossi et al. 1993).

1.5.1.2 *Pleurotus ostreatus* (Oyster Mushroom)

It is cultivated in China, and carbohydrate contents of oyster mushrooms are known to range from 5 to 6.7 g per 100 g fresh weight (Baars 2017). It is rich source of fiber, minerals, protein, and vitamins. It possesses unique flavor as well as aromatic properties and is used in different Chinese, Japanese, and Korean cookery as a source of delicacy. Adding more, it also possesses antibacterial, antibiotic, antitumor, antiviral, hematological, hypocholesterolemic, and immunomodulation activities (Cohen et al. 2002; Ghorai et al. 2009).

1.5.1.3 *Volvariella volvacea* (Straw Mushroom)

Volvariella volvacea is cultivated throughout East and Southeast Asia. It is natural source of antioxidants. It consists of fungal immunomodulatory protein FIP-vvo which is known to induce TH1- and TH2-specific cytokines (Cheung et al. 2003; She et al. 1998).

1.5.1.4 *Auricularia* sp. (Wood Ear Mushroom)

It is found worldwide. It is a rich source of magnesium, phosphorus, potassium, and selenium and has high dietary fiber content. It helps in relieving constipation. The fruiting body produces an immunomodulatory protein (APP) which enhances the production of nitric oxide and tumor necrosis factor-alpha (TNF-α), suggesting that APP is an immune stimulant. Further, APP is known to activate murine splenocytes, distinctly enhancing their proliferation and interferon gamma (IFN-γ) secretion (Kim et al. 2004; Sheu et al. 2004).

1.5.1.5 *Boletus edulis* Group (Porcini Mushrooms)

It is widely distributed in the Northern Hemisphere across Asia, Europe, and North America. These are good source of vitamins, minerals, and dietary fiber, and fresh mushrooms are known to consist of 80% moisture (Ouzouni and Riganakos 2007). The total lipid, or crude fat, content makes up 2.6% of the dry matter of the mushroom. The fruiting bodies consist of about 500 mg of ergosterol per 100 g of dried mushroom (Mattila et al. 2002) and about 30 mg of ergosterol peroxide per 100 g of dried mushroom. Ergosterol peroxide is known to exhibit antimicrobial and anti-inflammatory activities and cytotoxicity to tumor cell lines in laboratory cultures (Krzyczkowski et al. 2009). It contains lectin which stimulates cells to begin cell division, and is also known to inhibit reverse transcriptase enzyme (Zheng et al. 2007). Furthermore, the fruiting bodies are also known to possess antioxidant activity due to presence of organic acids, tocopherols, phenolic compounds (Tsai et al. 2007), and alkaloids, the highest antioxidant activity being in the cap (Ribeiro et al. 2008).

1.5.1.6 *Agaricus bisporus*

Agaricus bisporus is by far the most commonly cultivated mushroom around the world and its cultivation has been described for the first time in France by Tournefort in 1707 (Baars 2017). In the mid-1970s, the *Agaricus* crop accounted for over 70% of total global mushroom production (Moore and Chiu 2001). *Agaricus bisporus* is actually grown in two varieties, producing either white or brown mushrooms. It is rich in minerals including phosphorus, potassium, selenium, and sodium; further it is an excellent source of vitamin B especially riboflavin. Raw mushrooms are naturally cholesterol and fat free (Beelman et al. 2003).

1.5.2 Fungi as and in Processed Food

Fungi contribute a fair share of food and food additives in the markets as animal feed or human food. Fungi are utilized in the production of fermented food and beverages in all traditional and indigenous cultures in the world. Examples include beer, bread, cheeses, cider, rice, soy sauce, and wine (Dupont et al. 2016).

1.5.2.1 Mycoprotein

It is a form of single cell protein created from *Fusarium venenatum* (Wiebe 2002). Mycoprotein has been on sale to the public as Quorn since 1985 and is a popular meat substitute, particularly for vegetarians. It is produced by fermentation; the growth of the mold requires glucose, minerals, biotin, and ammonia. It has high fiber content which helps to decrease blood cholesterol levels (Turnbull et al. 1992; Wiebe 2004). Mycoprotein is a high-protein, high-fiber, low-fat food ingredient that is appropriate to be included in a healthy diet; it also reduces blood sugar levels additionally being a good source of zinc and selenium (Denny et al. 2008). Mycoprotein has been suggested to be used in the production of breakfast cereals and puffed snacks, or be added to yogurt and ice cream products as a fat replacer (Rodger 2001).

1.5.2.2 Cheese

Microorganisms play a major role in the cheese-making process, from the initial milk curdling by lactic acid bacteria to the maturation step by fungi such as yeasts and molds. In fact, microbiological and biochemical changes occurring during ripening have a direct influence on development of the texture as well as flavor that makes each kind of cheese unique. Primary biochemical changes include lipolysis that converts lactose into lactate and proteolysis that directly influences flavor through the production of short peptides and amino acids, originating from six

primary sources, secondary starters which include molds such as *Penicillium roqueforti* in blue cheeses and so on (McSweeney 2004). Secondary biochemical changes include metabolism of fatty acids and of amino acids by molds such as *Penicillium roqueforti* in blue cheeses and *Penicillium camemberti* or *Geotrichum candidum* in soft cheeses such as Camembert and Brie.

1.5.2.3 Blue Cheese

Penicillium roqueforti is used for the production of the blue cheese. *Penicillium roqueforti* has occurred in blue cheeses since at least antiquity (Labbe and Serres 2004; Labbe and Serres 2009; Vabre 2015). The fungus was not actually inoculated during production of blue cheese; rather it appeared spontaneously. Blue cheese has a unique look of blue streaks found all throughout. The blue veins are due to addition of *Penicillium roqueforti* and *Penicillium glaucum* to the cheese making process. Further, blue cheese also has a distinctive flavor as well as aroma which arises from methyl ketones that are actually the metabolic product of *Penicillium roqueforti*.

1.5.2.4 Camembert Cheese

The production of camembert cheese requires *Penicillium camemberti*. The mold is responsible for soft and buttery texture of cheese (Michelson 2010). The aqueous suspension of the *Penicillium camemberti* is sprayed on the surface of the cheese and kept for ripening for weeks, and this process of ripening gives cheese a distinctive bloomy, edible rind and creamy interior texture characteristic (Smith 2005). *Penicillium camemberti* is also used in flavoring of other foods including dry, fermented sausages. Besides *Penicillium roqueforti* and *Penicillium camemberti*, there are other important fungi that are used in cheese makings such as *Sporendonema casei* which is used for the production of Cantal and Salers. Some *Scopulariopsis* species including *Scopulariopsis candida*, *Scopulariopsis flava*, and *Scopulariopsis fusca* are found in uncooked hard cheeses. *Fusarium domesticum* is inoculated for the production of Saint Nectaire and Reblochon (Dupont et al. 2016). Further, *Mucor circinelloides*, *Mucor lanceolatus*, and *Mucor racemosus* are used for the production of uncooked hard cheeses including Saint Nectaire and Tomme de Savoie (Hermet et al. 2012).

1.5.2.5 Soy Sauce

Soy sauce has its origins in the Orient, but now it is popular around the world. It is produced by fermentation which involves use of *Aspergillus oryzae*, *Aspergillus sojae*, *Saccharomyces rouxii* and acetic acid bacteria to produce flavoring liquid with good nutritional qualities. In production of soy sauce, soybeans are soaked, cooked, mashed, and fermented with *Aspergillus oryzae* and *Aspergillus sojae* (Moore and Chiu 2001).

1.5.2.6 Indonesian Tempeh and Ang-kak

It is a white cake which involves the use of *Rhizopus oligosporus*. It is produced by fermentation of partially cooked soybean cotyledons with *Rhizopus oligosporus*. The fungus binds the soybean mass into a protein-rich cake which can be used as a substitute of meat and is widely sold into the vegetarian market (Moore and Chiu 2001). It is also known as red yeast rice and is popular in China and the Philippines. It is fermented using *Monascus purpureus* (Moore and Chiu 2001).

1.5.2.7 Alcoholic Beverages

Saccharomyces cerevisiae is extensively used for the production of alcoholic beverages. There are different categories of alcoholic beverages such as those which are produced using fruit juices, those which are produced using starchy materials, and those produced using other plant materials (Carlile et al. 2001). Alcoholic beverages which are produced using fruit juices include cider, perry, and wine. Yeasts convert sugars into ethanol and carbon dioxide under both anaerobic fermentation and aerobic conditions known as the Crabtree effect (Piškur et al. 2006; Hagman et al. 2013); due to this capability yeasts were in fact primarily used as effectual ways to preserve the quality as well as safety of the foods and beverages as high concentrations of ethanol are toxic for most of the other microbes.

Wines are produced by fermenting red and white grapes. The fermentation for wine production is a complex process which involves numerous genera and species of yeast that are part of the grape berries microflora (Pretorius 2000). The very first stage of fermentation consists of mainly non-*Saccharomyces* yeasts. Nevertheless, due to outstanding fermentative capabilities in anaerobic conditions and high tolerance to ethanol, *Saccharomyces cerevisiae* speedily dominates alcoholic fermentation which degrades majority of sugars in alcohol. The fermentation of wine consists of a lag phase and a short growth phase followed by a stationary phase, during which 50 to 80% of sugars is fermented (Dupont et al. 2016). Nitrogen has been considered to be the main limiting nutrient which is responsible for cell proliferation arrest though the availability of other micronutrients, for instance, lipids and vitamins can also be a limiting factor (Sablayrolles 2008). Nutrient imbalance may affect yeast fermentation ability, which may further result in stuck or sluggish fermentations, and can also influence the production of volatile compounds and the organoleptic balance of wine.

Cider is produced using apples and is popular in the UK. In fact, the UK has the world's highest per capita consumption, as well as the largest cider-producing companies. It is also popular in Australia, Canada, India, and New Zealand. The selection of yeast for production of cider is very crucial for the quality of the final product. Generally, there are categories of yeast which are utilized for cider production including commercially developed strains and wild, or autochthonous, strains. In either case, the species tend to be either *Saccharomyces cerevisiae* or *Saccharomyces bayanus*. In fact, the population of wild yeast could be amazingly diverse and commonly include the species of *Candida*, *Hanseniaspora*, *Metschnikowia*, *Pichia*, and

Saccharomyces (Valles et al. 2007). Characteristically, the native yeast takes up residence in the cidery and can be important to the unique flavor of the product (Bedriñana et al. 2010).

1.6 Value-Added Products from Fungi

The constant growth of agricultural production and development of novel mechanical processing technologies have also led to production of a variety of wastes, which are not easy to treat and valorize (Cheirsilp et al. 2011; Saenge et al. 2011). Wastewater generation is a continuous process in agro-industrial plants which creates disposal problems as well as is also a threat for the environment (Avancini et al. 2007). Furthermore, one-third of the food produced in the world for human consumption about 1.3 billion tons end up as waste every year. Twenty percent of dairy products end up as waste in agriculture, postharvest, processing, distribution, and consumption (Mahboubi et al. 2017). Thus, economically as well as ecologically either the reutilization or the valorization of wastes into high value-added products is of great importance (Balasubramanian et al. 2011). There are many instances where fungi have been used for the generation of diverse value-added products. Fungal bioconversion, through fermentative processes, has been revealed to be an eco-friendly biotechnological approach for the sustainable development of protein-rich animal feedstock (Dias et al. 2018; Jin et al. 2016; Salgado et al. 2015). Table 1.3 represents value-added products from fungal communities.

Winery by-products are known as low-cost substrates for production of pigments, such as carotenoids. Buzzini and Martini (2000) reported a maximum yield of carotenoids by cultures of *Rhodotorula glutinis* using grape must as the sole carbon source. Grape pomace, by-product from the wine industry, proved to be a good substrate that induced the production of commercially important hydrolytic enzymes including xylanases, pectinases, cellulases, using *Aspergillus awamori* (Botella et al. 2005).

Muniraj et al. (2015) showed the production of microbial lipids and γ-linolenic acid by *Aspergillus flavus* and *Mucor rouxii*, with potato processing wastewater as nutrient source. The mixed culture of *Aspergillus niger*, *Penicillium chrysogenum*, *Penicillium citrinum*, and *Trichoderma harzianum* in medium containing post-fermentation grape wastes as the only carbon source under submerged and solid-state fermentation conditions (Karpe et al. 2015b) produced commercially important metabolites. Further, *Penicillium chrysogenum* was capable of metabolizing pentoses into arabitol and xylitol, could degrade tannins and lignin, and could produce medicinally important metabolite, for instance, syringate (Karpe et al. 2015a). Biological surfactants are another type of value-added products that can be obtained from solid-state fermentation of grape wastes, using fungal species such as *Pleurotus djamor* (Velioglu and Urek 2015). Jin et al. (2016) found that *Aspergillus oryzae* and *Trichoderma reesei* yielded the highest protein enrichment and digestibility of the grape marc and lees in solid state fermentation. In the study of Martínez et al.

Table 1.3 Value-added products from fungal communities

Fungi	Substrate	Value added product	References
Aspergillus awamori	Grape pomace	Xylanases, pectinases, cellulases	Botella et al. (2005)
Aspergillus flavus	Wastewater	γ-Linolenic acid	Muniraj et al. (2015)
Aspergillus niger	Molasses and chicken feather peptone	Citric acid	Ozdal and Kurbanoglu (2018)
Aspergillus oryzae	Dairy waste	Biomass and ethanol	Mahboubi et al. (2017)
Aspergillus oryzae	Ethanol plant by-products	Ethanol and protein	Bátori et al. (2015)
Aspergillus uvarum	Lignocellulosic residues	Cellulases	Salgado et al. (2015)
Aspergillus uvarum	Lignocellulosic residues	Xylanases	Salgado et al. (2015)
Coriolus antarcticus	Grape stalk	Laccase, Mn-peroxidase activities	Levin et al. (2012)
Debaryomyces nepalensis	Grape stalk	Bioethanol	Egüés et al. (2013)
Irpex lacteus	Pretreated wheat straw	Ethanol	López-Abelairas et al. (2013)
Kluyveromyces marxianus	Sugarcane bagasse and sugar beet molasses	Alcohols	Martínez et al. (2017)
Kluyveromyces marxianus	Sugarcane bagasse and sugar beet molasses	Esters	Martínez et al. (2017)
Monascus purpureus	Orange processing wastes	Pigment	Kantifedaki et al. (2018)
Monascus ruber	Sugarcane bagasse hydrolysate	Red pigment	Hilares et al. (2018)
Mortierella isabellina	–	Oils	Carota et al. (2018)
Mucor rouxii	Wastewater	γ-Linolenic acid	Muniraj et al. (2015)
Neurospora intermedia	Dairy waste	Biomass and ethanol	Mahboubi et al. (2017)
Neurospora intermedia	Ethanol plant by-products	Ethanol and protein	Bátori et al. (2015)
Penicillium purpurogenum	Orange processing wastes	Pigment	Kantifedaki et al. (2018)
Phanerochaete chrysosporium	Grape seeds and barley bran	Lignin peroxidase and manganese-dependent peroxidase activities	Moredo et al. (2003)
Pleurotus eryngii	Pretreated wheat straw	Ethanol	(López-Abelairas et al. 2013)

(continued)

Table 1.3 (continued)

Fungi	Substrate	Value added product	References
Rhodotorula glutinis	Grape must	Carotenoids	Buzzini and Martini (2000)
Stereum hirsutum	Grape stalk	Endoglucanase	Levin et al. (2012)
Trametes hirsuta	Grape seeds	Laccase	(Couto et al. 2006)
Trametes trogii	Grape stalk	Endoxylanase	Levin et al. (2012)
Trametes versicolor	Lignocellulosic residues	Laccase	Moredo et al. (2003)
Trichoderma pseudokoningii	Cassava residue	Protein enrichment	Bayitse et al. (2015)

(2017), alcohols and esters were produced using strain of *Kluyveromyces marxianus* by valorization of sugarcane bagasse and sugar beet molasses. Carota et al. (2018) assessed the oil-producing performance of *Aspergillus* sp., *Cunninghamella* sp., *Mortierella* sp., and *Mucor* sp. *Mortierella isabellina* was found to be the most efficient among all the strains used. Further, the fatty acid analysis of the oils produced confirmed that they were apt for biodiesel production and exhibited high similarity to palm and *Jatropha* oils. Thus, the use of fungi can be one of the solutions for the treating waste and converting them into value-added products either for human consumption or to be used in animal feed as well as chemicals could also be produced as alcohols, esters.

1.7 Conclusion and Future Prospects

The indiscriminate and disproportionate use of chemical fertilizers is leading to health and environmental hazards as well as greatly affecting the agricultural productivity. To avoid the use of chemical fertilizers, alternative strategies are essential to protect the environment as well as for sustainability. In this regard, fungi with multifarious plant growth-promoting traits are environmentally safe as well as natural alternatives to replace chemical fertilizers. Thus, these fungi can be used as biofertilizers and even could be utilized under stress conditions. No doubt, more detailed studies are still required on how much of inoculum will be required, what will be the effect of cultivar on inoculum, will inoculum be able to survive under adverse conditions, and what will be the role of environmental conditions in altering the activity of inoculums. Further field experiments will finally reveal their applicability. Fungi not only act as potent bioinoculants but are also industrially important as they are the most preferred sources of enzymes for diverse industries including food, textile, pharmaceutical, baking, detergent, dairy, and so on. Fungi are a great resource pool for agriculture and industrial sector and for value-added products, and

a number of species have already been exploited for new generation of bio-based products. Thus, fungi are gaining attractiveness in the context of a global need as novel sources of food, enzymes, secondary metabolites, and many more.

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Chapter 2
Fungal Phytases: Biotechnological Applications in Food and Feed Industries

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2.1 Introduction

Technological advancement with state-of-the-art facilities and economic demands are driving firm toward developing new food and feed products in the market. Enzymes are one of the key industrial products that received huge demand worldwide. The global enzymes' market size was estimated to be USD 8.18 billion in 2015 and is expected to undergo significant growth over the next 8 years driven by their growing application in detergents, pharmaceuticals, food, and beverages (Market Research Report 2016; El Enshasy et al. 2018). Nowadays, enzymes, e.g., amylases, invertases, xylanases, proteases, phytases, and many other enzymes, became major components during production processes in food and feed industries (El Enshasy and Elsayed 2017; El Enshasy et al. 2013, 2016; Elsayed and Danial 2018; Elsayed and El Enshasy 2018; Elsayed et al. 2016).

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Phytic acid, an important nutrient source of organic phosphorus, is synthesized in plants and stored in ripening seeds. However, due to its antinutritional characteristics, i.e., the tendency to form phytate complex with other nutrients, such as proteins, it is not easily to be digested by monogastric animals that lack enzymes responsible for phytate dephosphorylation (Othman et al. 2014). Consequently, such complexes will be excreted in animal manure causing nutrient deficiency in animals, and subsequently environmental pollution. Accordingly, phytases play an important role in overcoming these challenges.

Phytase enzymes account for about 60% of the enzyme market used for animal nutrition, about USD 350 million annually (Corrêa et al. 2015). Nowadays, phytases are commonly added to poultry diets to improve phosphorus utilization, leading to the reduction of feed cost and phosphorus pollution (Dersjant-Li et al. 2015). Phytases are phosphohydrolases that catalyze the hydrolysis of phytate to myoinositol derivatives and inorganic phosphate (Shanmugam 2018). Phytases can be found mainly in microbial and plant organisms. However, due to their ease of cultivation and higher productivity of extracellular enzymes, filamentous fungi are considered one of the best industrial phytase sources (Ocampo et al. 2012).

Ongoing research continues focusing on discovering novel phytase producers, utilizing recombinant microorganisms as well as improving production processes and efficient product recovery. Generally, industrial processes involving feed-pellet formation favors the utilization of thermostable phytases to avoid their inactivation at normal animal body temperatures (Corrêa et al. 2015; Ma et al. 2011).

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2.2 Phytic Acid

Phytic acid, or myo-inositol hexaphosphoric acid ($InsP_6$), is a natural compound abundant in many seeds and fruits, such as wheat, corn, rice, coconut, or pumpkin seeds (Diouf-Lewis et al. 2017). Phytic acid is also known as phytate salt and phytin. The molecular formula of phytic acid is $C_6H_{18}O_{24}P_6$ with a molecular weight of 660.03. Figure 2.1 illustrates the chemical structure of phytic acid.

Due to the characteristic chemical structure of phytic acid bearing high negative charges at physiological pH values, therefore, it easily precipitates as phytate salts through binding with different mineral cations, such as iron, zinc, potassium, calcium, and magnesium (Iwai et al. 2012). The complexes formed hereby adversely affect their absorption in the gastrointestinal tract (Tiwari and Singh 2012). Monogastric animals including humans lack phytases in their digestive tract, and are therefore unable to process phytates present in seeds. Consequently, phytic acid is not efficiently digested and the nutritional values of seeds are limited through phosphorus and minerals (Cominelli et al. 2018). This in turn affects the common practice of feeding with high phosphate nutrients due to its fecal excretion and increases environmental pollution as well.

In plant seeds, phytic acid is the most abundant form of phosphorus, accounting for about 85% of total phosphorus, with amounts even 1000-fold higher than those detected in vegetative and other plant parts, such as pollen, roots, tubers, and turions (Sparvoli and Cominelli 2015). Phytate accumulates within protein bodies, generally of vacuolar origin, in seed storage cells and is usually concentrated in spherical inclusions called globoids (Iwai et al. 2012). It plays an essential role in relation to environmental stress and hormonal changes and as a backup source for phosphorus and energy (Cominelli et al. 2018).

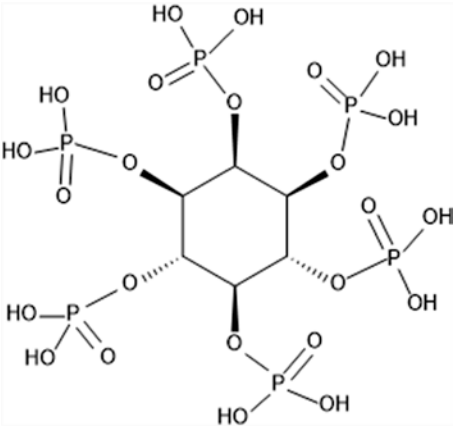


Fig. 2.1 Phytic acid molecular structure

2.3 Classification of Phytase

Phytases can be classified into different classes according to a number of criteria including stereospecificity of phytate hydrolysis (carbon number in the myoinositol ring of phytate at which dephosphorylation is initiated), optimal pH (alkaline or acidic phytases), and its catalytic mechanism. In terms of catalytic mechanism, phytases can be classified into four groups which are histidine acid phytases (HAPs), ß-propeller phytases (BPPs), cysteine phytases (CPs), or purple acid phosphatase (PAPs). Depending on their optimum pH, phytases can be divided further into acidic and alkaline phytases, while based on their stereospecificity, another three groups can be identified as 3-phytases (E.C. 3.1.3.8), 6-phytases (E.C. 3.1.3.26), and 5-phytases (E.C. 3.1.3.72; Greiner and Konietzny 2010). Table 2.1 summarizes different structural divisions of phytate-degrading enzymes based on mechanistic enzymology.

2.3.1 Histidine Acid Phytases

This phytase subfamily includes most of the currently identified phytases which can work independently without requiring the presence of cofactors for their optimal activity (Greiner and Konietzny 2010). Nowadays, 48 phytase structures from all subfamilies, except Purple Acid Phosphatase, have been deposited in the Protein

Table 2.1 Structural classes of phytate degrading enzymes

Enzyme family	Unique structural feature	Catalytic mechanism/adaptation to hydrolyzes phytate	Examples
Histidine acid phosphatase	N-terminal RHGXRXP C-terminal HD consensus motif	N-terminal H forms a phosphohistidine intermediate, C-terminal acts as proton donor/ Substrate specificity site residues positively charged	*A. niger* *Peniophora lycii* *E. coli* *Zea mays L.*
β-propeller phytase	Six-bladed propeller shaped molecule	Mechanism consists of an affinity site and a cleavage site. Affinity sites bind phosphate group, while other sites attack adjacent phosphate group/dual site favors IP6, IP5, or IP4 as substrate	*Bacillus* sp. *X. oryzae*
Cysteine phosphatase	Phosphorous loop structure contains HCXXGXXR(T/S) consensus motif	Protein tyrosine phosphatase mechanism cleaves phosphate groups/Deeper active site pocket accommodates phytate	*S. ruminantium*
Purple acid phosphatase	Consensus motif: DXG/ GDXXY/GNH (E, D)/ VXXH/GHXH	Metalloenzymes, phylogenetically linked to large plant PAP/unknown	*Glycine max* *Medicago truncatula*

Adapted from Lei et al. (2007)

Data Bank (PDB) including 25 HAPs (Chen et al. 2015). HAPs are acidic phytases that are able to function properly in the gastrointestinal tracts of swine and poultry. HAP structure consists of two folds, which are a larger α/β-domain and a smaller α-domain. The α/β-domain consists of a central six-stranded β-sheet, which is surrounded by two α-helices on each side, while the α-domain consists of five major α-helices and several short helices (Table 2.2). The consensus active-site motifs of RHGXRXP and HD are located in a substrate-binding pocket, which lies in the domain interface. Under the optimal operating conditions, HAP members exhibit higher efficacy (specific activity, 100–>3000 U/mg at pH 2.5–7.5). On the other hand, they are unstable at temperatures >65 °C. Remarkably, *Aspergillus fumigatus* produces a phytase belonging to HAPs group, which has an outstanding heat-resilient property, >80% residual activity after being heated at 100 °C for 20 min (Chen et al. 2015).

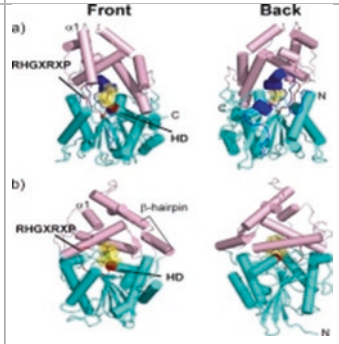
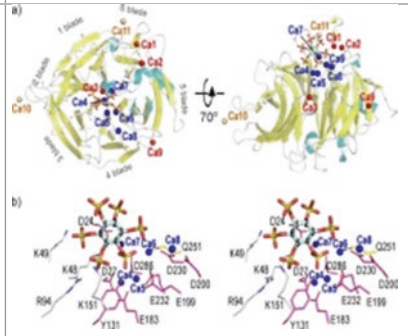
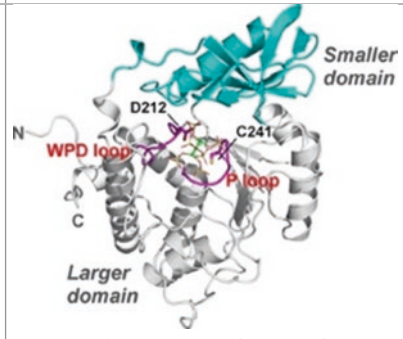
2.3.2 β-propeller Phytases (BPPs)

β-propeller phytases, also known as alkaline phytases, play an important role in phytate-phosphorus cycling in soil and aquatic environment (Huang et al. 2009). These enzymes exploit Ca^{2+}-dependent catalytic mechanisms with strict substrate specificity for hydrolytic reaction and protein thermostability (Korsmeyer et al. 2000). BPPs consist of a six-bladed propeller folding architecture with six calcium-binding sites in each protein molecule. The substrate binding site is present on the top of the β-propeller. Binding of three calcium ions to high-affinity calcium binding sites dramatically increases the thermal stability of the enzyme by joining loop segments found remote in the amino acid sequence. Furthermore, the catalytic activity is turned on by the binding of three additional calcium ions to low-affinity calcium binding sites at the top of the molecule by converting the highly negatively charged cleft into a favorable environment for phytate binding (Greiner and Konietzny 2010). The blades are aligned to surround the central tunnel, in which many bound water molecules were identified. Generous hydrophobic interactions found between the blades are believed to stabilize the overall protein folding (Chen et al. 2015).

2.3.3 Protein Tyrosine Phosphatase-Like Phytases (PTPLPs)

The PTPLPs, also regarded as cysteine phytases or cysteine phosphatases, emulate the protein fold and catalytic mechanism of tyrosine phosphatase. High hydrolytic activity is performed toward phytate at optimal pH ranging from 4.0 to 6.0 (Chen et al. 2015). The PTPLP proteins fold into a larger and a smaller domain. The larger one consists of a 4-β-sheet, which is sandwiched by several α-helices on both sides, while the smaller domain mainly consists of a 5-stranded β-sheet.

Table 2.2 Phytases subfamily structures (Chen et al. 2015)

Subfamily	HAPs	BPPs	PTPLPs
Structure	 The front and back views of HAP structures from (a) *A. niger* (PDB ID: 1IHP) and (b) *E. coli* (PDB ID: 1DKN). The α-domains, α/β-domains, and N-terminal region in the AnPhyA, pink, cyan, and blue, respectively; RHGXRXP and HD motifs, red; IHS in AnPhyA and IHP in EcAppA, stick models	 Top and side views of the ternary structure of *B. subtilis* alkaline phytase (PDB ID: 3AMS); β-strand, yellow; α-helix, cyan; loop, white; Ca1–3 and Ca9 (protein stability), red; Ca4–8 (catalytic reaction), blue; Ca10–11 (crystal packing), orange, side chains of residues (direct and indirect interactions to the IHS); gray and magenta, Q251 (direct and indirect interactions), yellow	 The complex structure of PTPLP from *S. ruminantium.* The large and small domains: gray and cyan (PDB ID: 1 U26) respectively; the IHS and the side chains of the catalytic residues: line and stick models; the loops that accommodate catalytic residues: magenta

2.3.4 Purple Acid Phosphatase (PAP)

PAPs produced by plants, mammals, fungi, and bacteria contain binuclear Fe (III)-Me(II) centers, where Me is Fe, Mn, or Zn. To date, purple acid phosphatases with phytase activity appear to be only found in plants (Yao et al. 2012). Unlike HAPs, BPPs, and PTPLPs, X-ray crystallography studies have not yet been performed for PAP phytases, and there is no information available on the adaptation of PAPs active site to phytate as a substrate. Table 2.1 shows the structural division of phytate degrading enzymes based on their mechanistic enzymology.

2.4 Production of Phytases by Different Biofactories

Phytases are widely distributed in nature and can be found in microorganisms (bacteria and fungi) as well as plants. The earliest initiative for development of phytase production took place in 1962 by International Minerals and Chemicals Co., which was accounted as the only available market producer until 1990s, after which phytase commercialization started on a large scale (Lei et al. 2013). The use of microbial phytases is generally more favored due to their economic feasibility, higher yields, consistency, ease of product modification and optimization, regular supply due to absence of seasonal fluctuations, rapid growth of microbes on inexpensive media, stability, and greater catalytic activity (Gurung et al. 2013). In addition, microbial phytases have broad pH spectrum of activity, where they are stable at pH as low as 3.0 and as high as 8.0, whereas stability of plant phytases decreases below 4.0 and above 7.5 (Balwani et al. 2017). Although many phytase products, particularly those from fungal sources, are now commercially available in the market, continuous search for novel phytases has been driven by the encountered limitations of some phytases, namely, substrate specificity, lower thermostability, lower resistance to proteolysis and acidity, as well as catalytic inefficiency (Tan et al. 2015).

Various types of bacterial species have been reported as phytase producers (Kumar et al. 2016; Kumar et al. 2017). These bacteria include *Enterobacter aerogenes* (Muslim et al. 2018), *Mitsuokella jalaludinii* (Tan et al. 2015), *Klebsiella pneumoniae* 9-3B (Escobin-Mopera et al. 2012), *Bifidobacterium* spp. (Tamayo-Ramos et al. 2012), *Bacillus* spp. (Kumar et al. 2013a; Sreedevi and Reddy 2012), *Geobacillus* spp. (Dokuzparmak et al. 2017), and *Selenomonas ruminantium* (Yanke et al. 1999). Moreover, several bacterial species were used as a host for expressed phytase genes such as *Lactobacillus casei* (García-Mantrana et al. 2016), *Escherichia coli* (Lan et al. 2014), and *Kluyveromyces lactis* (Ushasree et al. 2014). Production of bacterial phytases has been stimulated using metal ions like Cu^{2+}, Fe^{2+}, Mg^{2+}, Fe^{2+}, and Al^{2+} (Dokuzparmak et al. 2017).

Over the years, numerous efforts have been carried out to isolate and screen potential phytases from yeasts. One study reports about the isolation and screening of 600 yeast strains for phytase production, resulting in the selection of five isolates (*Zygosaccharomyces bisporus* NCIM 3265 and 3296, *Williopsis saturnus* NCIM 3298, *Z. priorionus* NCIM 3299 and *Schizosaccharomyces octosporus* NCIM 3297) as potential phytase producers (Pable et al. 2014). Other phytase-producing yeasts include *Saccharomyces cerevisiae* (Klosowski et al. 2018), *Schwanniomyces occidentalis*, and *Candida parapsilosis* (Ranjan and Sahay 2013).

Several studies focused on fungi as higher phytase producers in comparison to bacteria and plants. Such fungal strains were found to produce the most active extracellular phytase having with the most suitable characteristics of both pH and temperature stabilities. Phytases used in food and feed industrial applications should be resistant to the action of the proteases present in the intestinal tract. Fungi reported as phytase producers include *Rhizomucor pusillus* (Chadha et al. 2004), *Aspergillus ficuum* (Coban and Demirci 2014), *Penicillium oxalicum* (Kaur et al. 2017), and *Thermomyces lanuginosus* (Berka et al. 1998). Furthermore, *A. niger* has been found not only attractive for its generally recognized as safe (GRAS) status for use in food processing by the US Food and Drug Administration (US-FDA), but also for its ability to produce highly active phytase extracellularly. Recent interests have increased on research with thermophilic fungi, because they were able to secrete unique phytases at higher temperature optima, with higher organic solvent tolerance and long shelf life (Riyadi et al. 2017; Cassia Pereira et al. 2015).

Plants have been considered as one of the alternative expression systems for phytase production. Plants as biofactories become interesting among researchers and industries due to their ability to be transformed as cheap protein sources. Phytase genes from various microbial sources have been overexpressed in different plants including rice (Wang et al. 2017), *Arabidopsis* roots (Belgaroui et al. 2016), maize (Chen et al. 2013), rapeseed (Wang et al. 2013), and sesame (Jin et al. 2005). The *phy* genes from fungi have been extensively employed in transformation studies since they exhibit better stability in a wide range of pH and temperatures (Gontia et al. 2012). In 1993, engineering of stable and active recombinant phytase from *A. niger* in tobacco seeds was first reported (Pen et al. 1993). The fungal phytase gene (*phyA*) was fused to a plant endoplasmic reticulum-targeting sequence and was placed under the control of the constitutive 35S cauliflower mosaic virus (CaMV) promoter in a binary transformation vector. Another study shows the possibility to reduce costs by overexpression phytase in plant roots that will be able to consume soil phytate, thus reducing agricultural costs and minimizing phytate levels in agriculture intensive soil (Kumar et al. 2010). Recently, research developed low phytate rice seeds by silencing *IPK1* gene (Ali et al. 2013). In such manner, substantial reduction in seed phytate levels was observed without hampering the growth and development of transgenic rice plants. Table 2.3 shows the list of phytase-producing microbes (bacteria and fungi) and plant.

Table 2.3 List of phytase-producing microbial and plant

Phytase source	Species name	Reference
Bacteria	*Acinetobacter baumannii*	Alias et al. (2017)
	Acromobacter sp.	Kumar et al. (2013a)
	Advenella sp.	Singh et al. (2014)
	Bacillus cereus	Dan et al. (2015)
	Bacillus coagulans	Irwan et al. (2017)
	Bacillus subtilis	Rocky-Salimi et al. (2016)
	Bacillus amyloliquefaciens	Olajuyigbe (2016)
	Bacillus licheniformis	Dan and Ray (2014)
	Bacillus stearothermophillus	Irwan et al. (2017)
	Cellulosimicrobium sp.	Singh et al. (2014)
	Citrobacter freundii	Zhao et al. (2010)
	Echerichia coli	McKinney et al. (2015)
	Enterobacter aerogenes	Muslim et al. (2018)
	Geobacillus sp.	Jorquera et al. (2018)
	Klebsiella aerogenes	Escobin-Mopera et al. (2012)
	Klebsiella terrigena	Greiner and Carlsson 2006
	Klebsiella oxytoca	Jareonkitmongkol et al. (1997)
	Lactobacillus casei	García-Mantrana et al. (2016)
	Lactobacillus panis	Nuobariene et al. (2015)
	Lactobacillus plantarum	Sumengen et al. (2013)
	Mitsuokella jalaludinii	Tang et al. (2017)
	Pseudomonas sp.	Muslim et al. (2018)
	Staphylococcus caprae	Dan et al. (2015)
	Tetrathiobacter sp.	Kumar et al. (2013b)
	Weissella kimchii	Andrabi et al. (2016)
Fungi	*Aspergillus niger*	Saithi and Tongta (2016)
	Aspergillus carneus	Ghareib (1990)
	Aspergillus flavus	Gaind and Singh (2015)
	Aspergillus fumigatus	Gangoliya et al. (2015)
	Aspergillus ficuum	Wang et al. (2011)
	Aspergillus oryzae	Sapna (2014)
	Aspergillus tubingensis	Qasim et al. (2017)
	Mucor racemosus	Bogar et al. (2003)
	Myceliophthora thermophila	Mitchell et al. (1997)
	Penicillium purpurogenum	Awad et al. (2014)
	Rhizomucor pusillus	Chadha et al. (2004)
	Rhizopus oligosporus	Suresh and Radha (2015)
	Rhizopus oryzae	Rani and Ghosh (2011)
	Trichoderma viride	Aseri et al. (2009)
Plant	Rice	Wang et al. (2017)
	Maize	Chen et al. (2013)
	Rapeseed	Wang et al. (2013)

(continued)

Table 2.3 (continued)

Phytase source	Species name	Reference
	Sesame	Jin et al. (2005)
	Arabidopsis roots	Belgaroui et al. (2016)

2.5 Phytase Production

2.5.1 Production of Phytase by Recombinant Microorganisms

Fungal phytases have been isolated from over 200 fungal cultures of *Aspergilli*, *Mucor*, *Penicillium*, and *Rhizopus* species (Liu et al. 1998). However, due to the increasing biotechnological and industrial importance of phytases, recent years showed significant progress in the optimization of phytase production in terms of molecular biology and biochemical tools (Singh and Satyanarayana 2011). Cloning and recombinant DNA technology have been used to enhance commercial production of fungal phytases.

Traditionally, Pasamontes et al. (1997) were first able to clone two phytases from *Talaromyces thermophilus* and *Emericella nidulans*, and found that the enzymes consisted of 463 and 466 amino acids, respectively, and showed higher similarity to normal wild phytases. Furthermore, the gene encoding for extracellular phytase was cloned from *T. lanuginosus* and was expressed in *Fusarium venenatum* (Berka et al. 1998). They found that the activity of *T. lanuginosus* phytase was maintained at 75 °C, with higher catalytic properties than any fungal phytase at 65 °C as optimum temperature. Trials have been further made to engineer phytase characteristics depending on the field of application. Due to the absence of thermostable natural phytases for feed additives, comparisons of amino acid sequences have been used as a technique for designing and preparing consensus phytases (Lehmann et al. 2000). Such comparisons using 13 fungal phytase sequences made it possible to produce an engineered phytase, not only with normal enzymatic characteristics but also with an improved thermostability, where the unfolding temperature increased by about 15–20 °C. Accordingly, it was possible to compare crystal structure of such consensus phytase with its *A. niger* counterpart. The fact that *A. niger* phytases have lower thermostability leads to the discovery that there is a direct relationship between protein sequence conservation and phytase stability (Singh et al. 2018a). Recently, Li et al. (2011) were able to clone a 2060-bp-long sequence from rice (*Oryza sativa* L.) which produced a phytase enzyme of 519 amino acids. Furthermore, the gene of *A. niger* phytase was expressed in *S. cerevisiae* to investigate the effects of glycosylation on phytase activity and thermostability (Han et al. 1999). Authors incorporated a 1.4 kb DNA segment encoding the *phyA* gene into the expression vector pYES2 and expressed it in *S. cerevisiae* for producing extracellular phytase. They found that medium composition and signal peptide significantly influenced the activity of the produced extracellular phytase. Phillipy and Mullaney (1997) found that the produced phytase lost about 9% and 40% of its activity and thermostability,

respectively, upon deglycosylation. Additionally, *Pichia pastoris* has been used as a host strain for expressing phytase genes (*phyA*) from *A. niger* and *Bacillus subtilis* (Han et al. 1999; Guerrero-Olazarán et al. 2010). Both works produced high amounts of active extracellular phytases. Furthermore, *P. pastoris* has been used for cloning and expressing phytase gene (*phyA2*) through excising and removing signal peptide encoding sequence and intron sequence (Yao et al. 1998). Huang et al. (2008) were able to produce a recombinant pH-resistant and thermostable phytase in *P. pastoris* by cloning and expressing phytase gene from *Yersinia kristensenii* having a protein of 441 amino acids with 24 amino acid signal peptide.

The discovery of modern genetic engineering techniques revolutionized research on phytase production. Such techniques included genome mining, functional metagenomics, cDNA cloning, and PCR variants (Vasudevan et al. 2017). Ma et al. (2011) used PCR techniques to isolate a novel gene for thermostable phytase (*PhyA*) from *A. aculeatus* RCEF 4894. They expressed the gene in *P. pastoris* with a specific phytase activity of 3000 U/mL at pH 5.5. Furthermore, the expressed phytase showed higher thermostability, where it was able to survive up to 90 °C for 10 min. The full-length gene comprised 1404 bp and encoded 467 amino acid residues with a 19-residue putative N-terminal signal peptide. Li et al. (2005) combined genetic modification strategies to enhance phytase production from *Citrobacter amalonaticus* in *P. pastoris*. They combined strategies as modification of P_{AOX1} promoter, choice of appropriate signal peptide, and augmentation of the gene dose. The modification of the first two parameters led to enhancing phytase yield by 35% and 12%, respectively. Furthermore, yield increased by about 141% upon increasing copy number of the *Phy* gene to six. Recently, Tang et al. (2018) applied directed evolution and site-directed mutagenesis to improve the thermostability and activity of recombinant phytase from *A. niger* N25. Their characterization and structural analysis results clearly demonstrated that the obtained mutations were able to produce cumulative or synergistic improvements in terms of enzyme thermostability or catalytic efficiency. Singh et al. (2018b) used molecular modeling and docking to investigate the molecular and biochemical properties of expressed recombinant thermophilic phytase from *Sporotrichum thermophile*. They found that the recombinant enzyme showed broad substrate specificity, and enzyme docking with inhibitors showed differential binding with GoldScore values ranging from 22.94 (2,3-butanedione) to 85.72 (myoinositol hexasulfate). Also, phytase docking with metavanadate showed binding at the same atom in the active site where the substrate binds.

Generally, it has been revealed that the bottleneck point in directed evolution for improving thermal stability of designed and produced phytase depends largely on the selection of the "right" method for mutagenesis and screening. As mentioned above, several phytases have been designed and produced for their higher thermostability. This depended mainly on the substitution of the majority of amino acid sequences that are chemically different in their respective sequences in the wild-type strains (Kim et al. 2008; Kim and Lei 2008). Such chemical diversity correlates well with mutagenesis methods applied to generate diverse mutational spectra enriched with functional traits. For example, investigating different epPCR libraries

failed to produce a promising thermostable variant from *Y. mollaretii*. On the other hand, applying high mutational load to generate a chemically diverse library resulted in the production of a thermostable phytase variant M1 with only moderate screening efforts (Shivange et al. 2011). Therefore, such results proved to provide a clear idea about employing different mutagenesis methods to generate chemically diverse substitutions, which in turn will enhance and enrich the obtained phytase variant libraries accompanied with reducing screening molecular efforts.

2.5.2 Production Methods Using Submerged Fermentation (SmF) and Solid-State Fermentation (SSF)

Phosphorus is one of nature's paradoxes as it is life's bottleneck for subsistence on earth but at the same time is detrimental in surplus quantities in an aquatic environment. Phytase is likely to play a critical role in the dephosphorylation of antinutritional and indigestible phytate, a phosphorus locking molecule, to digestible phosphorus, calcium, and other mineral nutrients in the coming future. However, the production of phytase has several limitations, such as diluted enzyme concentration, extensive downstream procedures, and treatment of generated effluents. The process is also expensive, time consuming, and difficult to scale up. Hence, efforts are required to produce cost effective phytases with fast upstream and economic downstream processing (Bhavsar and Khire 2014).

The production levels of phytase in naturally occurring strains are very low to be economically viable. Improvement in phytase production is achieved mutually by developing production technologies and engineered phytases. Strain improvement by mutagenesis and selection is a highly developed technique. It plays a vital role in the commercial development of microbial fermentation processes. Mutagenic procedures can be carried out in terms of type and dose of mutagen to obtain mutants, which may be screened for improved phytase, as seen in *A. niger*, using physical and chemical mutagenesis (Bhavsar et al. 2012). Another advanced method is the application of protoplast fusion, which has a significant potential for strain improvement and has been applied for various industrially important microorganisms. Protoplast fusion may be used to produce interspecific or even intergeneric hybrids, and is an important tool, since it can overcome the limitations of conventional mating systems in gene manipulation (Murlidhar and Panda 2000).

Although phytases are widely distributed in nature, the production in wild-type organisms is still far from being economically feasible. Accordingly, cloning and expression of phytase genes in suitable host organisms is necessary to obtain higher productivities. Since the cost effectiveness of phytase production is a major limiting factor for its application, therefore, different heterologous expression systems and hosts have been evaluated, such as plants, bacteria, and fungi (Bhavsar et al. 2012). Phytase production using fungal strains can be carried out using SmF or SSF. SmF is normally used in many processes for enzyme production. Phytases are mostly produced extracellularly and excreted into the fermentation medium. Production in

SmF provides better process control in terms of mass transfer, heat transfer, and oxygen supply. More than 75% of the industrial enzyme production is currently produced by SmF due to its ability to support the application of genetically modified microorganisms and the lack of paraphernalia compared to SSF (Subramaniyam and Vimala 2012). Additionally, SmF provides relatively low labor costs and low scale-up requirements when compared to SSF (Singhania et al. 2010). SmF technique, however, has several disadvantages, such as moderate product yields, higher costs, and the generation of significant amount of wastewater effluents (Abd-Elhalem et al. 2015). In SmF, substrate is a free flowing liquid or broth, which is used up rapidly and has to be constantly replenished (Irfan et al. 2016). Several types of bioreactors were screened for phytase production including stirred tank and air-lift bioreactors (Maller et al. 2014).

About 5000 years ago, fungi were cultivated in SSF to produce food, the oldest known rice fermentation by *A. oryzae*, in Koji production process (Shivanna and Venkateswaran 2014). Nowadays, this cultivation strategy is widely applied in various processes, such as bioremediation, biodetoxification of different hazardous compounds, production of various therapeutic enzymes and secondary metabolites, and as an effective alternative to SmF (Ashok et al. 2017). In SSF, a solid phase with minimal moisture content is used as a substrate for microbial growth. Recent studies reported that SSF is potentially a good alternative for SmF, as it provides higher quality and higher activity of extracts (Martins et al. 2011). Substrates are utilized slowly and efficiently over long fermentation periods (Chow and Ting 2015). The wastes generated after fermentation process can be recycled as a feed stock for other processes (Ballardo et al. 2016). Many examples of solid-state fermentation have been reported by researchers employing tray bioreactors, laterally aerated mixing beds, rotating drum bioreactors, packed bed bioreactors, and several other sophisticated systems. However, the main challenge in SSF is that the bioreactor design could not be upscaled to the required industrial level.

2.5.3 Factors Affecting Phytase Production

Several factors contributing to the effectiveness of phytase production process by fungi include, but not limited to, carbon source, nitrogen source, incubation time, temperature, pH, and inoculum size. In addition, the interactions between these factors affect the production process directly through the changes in enzyme biosynthesis, as well as indirectly by changing growth morphology, which is reflected on the physiological status and productivity of cells.

2.5.3.1 Carbon Source

Carbon source is an important nutrient for energy and cell growth. Studies conducted by Qasim et al. (2017) show that glucose addition to the fermentation medium significantly increased phytase production by *A. tubingensis* SKA

compared to other carbon sources tested, such as maltose, fructose, galactose, corn starch, and lactose at a concentration of 1.5%. Similar studies reported that 0.5% glucose was the most suitable for phytase production by *A. flavus* and *A. fumigatus* (Gaind and Singh 2015; Van Tinh et al. 2017). Furthermore, *Penicillium purpurogenum* GE1 was also reported to produce maximal phytase production using glucose as a carbon source under SSF (Awad et al. 2014).

2.5.3.2 Nitrogen Source

Nitrogen source plays a major role in cellular growth and phytase production by fungal strains. Among all nitrogen sources (organic and inorganic) tested at 1% (w/w) concentration (peptone, corn steep solids, urea, yeast extract, ammonium sulfate, ammonium nitrate, and sodium nitrate), ammonium nitrate was found to be the most suitable for maximal phytase production by *Rhizopus oryzae* culture (Suresh and Radha 2016; Ramachandran et al. 2005). Corn steep solids did not show any impact on phytase production, while inorganic nitrogen sources such as sodium nitrate and ammonium sulfate inhibited phytase biosynthesis (Ramachandran et al. 2005). However, Qasim et al. (2017) reported that among all nitrogen sources tested including $(NH_4)_2SO_4$, $NaNO_3$, NH_4Cl, peptone, yeast extract, and beef extract at concentrations of 0.5%, ammonium sulfate was used to maximize phytase production by *P. purpurogenum*. On the contrary, *P. purpurogenum* was reported to produce maximal phytase production using peptone as a nitrogen source under SSF (Awad et al. 2014). Other studies reported that 0.5% malt extract was a better choice for phytase production by *A. flavus* and *A. fumigatus* (Gaind and Singh 2015; Van Tinh et al. 2017).

2.5.3.3 Temperature

Temperature is considered as a crucial factor affecting cellular growth as well as enzyme production and stability by fungal strains. Previous results revealed the presence of linearity between phytase production and fermentation temperature by *A. niger* CFR 335 and *A. ficuum* SGA 01 up to 30 °C in both SmF and SSF (Shivanna and Venkateswaran 2014). As the fermentation temperature was increased to 60 °C, phytase production decreased by about 90% and 70% in SmF and SSF, respectively. Similar results showed that *A. tubingensis* SKA required an optimal incubation temperature of 30 °C in SSF (Qasim et al. 2017). On the other hand, *A. flavus* required an optimal temperature of 37 °C for phytase production under SSF (Gaind and Singh 2015). *P. purpurogenum* GE1 was reported to produce maximal phytase at 27 °C under SSF (Awad et al. 2014).

2.5.3.4 pH

Medium pH plays a vital role in the cellular growth and phytase production and stability. pH affects the ionization of the growth medium nutrients and, hence, directly influences enzyme production (Moreira et al. 2014). Previous results showed a direct relationship between medium pH and phytase production by *A. niger* CFR 335 and *A. ficuum* SGA 01 up to pH 4.5 in both SmF and SSF (Shivanna and Venkateswaran 2014). Above pH 4.5, the enzyme production was reduced by about 90%. Significantly high values of phytase activity by *A. tubingensis* SKA were obtained in SSF within a pH range of 4.5–5.5 (Qasim et al. 2017). For *A. flavus*, it was reported that the optimum production of phytase was pH 6.0 with drastic reduction in highly acidic pH environment (4.0) (Gaind and Singh 2015). Other types of fungi such as *P. purpurogenum* GE1 were reported to produced maximal phytase production at optimal pH 8.0 under SSF (Awad et al. 2014).

2.5.3.5 Cultivation Time

Cultivation period, time required for cells to grow and convert available nutrients into product, was found to significantly affect fungal growth and phytase production. Qasim et al. (2017) reported that *A. tubingensis* SKA produced phytase initially after 24 h of inoculation, and continuously continued enzyme production for 96 h, where it decreased after which. Similar observations were reported by previously for *A. oryzae* (Singh 2014). *Rhizopus oryzae* NRRL 1891 required an optimal incubation of 72 h to reach maximal phytase production (Ramachandran et al. 2005). The effect of incubation time on phytase production by *A. niger* CFR 335 and *A. ficuum* SGA 01 in SmF and SSF was investigated, where the optimal production was at 120 h, and decreased by about 70% when the fermentation continued to 240 h (Shivanna and Venkateswaran 2014). On the other hand, maximal phytase production by *A. niger* FM-32 was obtained after 13 days of cultivation using SmF (Toroğlu et al. 2015).

2.5.3.6 Inoculum Size and Age

The concentration of inoculum plays an important role in fungal phytase production. Several reports showed that different inoculum sizes gradually increased phytase production up to a certain level in both SmF and SSF. Shivanna and Venkateswaran (2014) reported that maximal phytase production was obtained with 0.5 and 1.0 mL of *A. niger* CFR 335 and *A. ficuum* SGA 01 spore suspensions (2×10^6 spores/mL) in SmF, respectively. On the other hand, SSF required 1 and 1.5 mL of *A. niger* CFR 335 and *A. ficuum* SGA 01 spore suspensions as optimal inoculum sizes, respectively. Moreover, 1×10^5 spores/mL was found to be optimal

inoculum size for optimal phytase production using *A. niger* USM AI1 and *A. tubingensis* using SSF (Qasim et al. 2017). Gaind and Singh (2015) found that an inoculum size of 6×10^5 cfu/mL, equivalent to 10.0% (w/v), was optimal for phytase synthesis, above which level, cell biomass increased on the expense of phytase production. Concerning inoculum age, it has been found that inoculum age strongly influences cell growth and fungal phytase production. Inoculum of 72 h-old culture of *A. flavus* was used as an optimal inoculum producing maximal phytase, while older inoculums resulted in a decreased phytase production (Gaind and Singh 2015). On the other hand, enzyme production rate was found to increase gradually with the increase in inoculum age up to 6 days and declined upon using older inoculums by both *A. niger* CFR 335 and *A. ficuum* SGA 01 under SmF and SSF, respectively (Shivanna and Venkateswaran 2014). Phytase productivity produced by *A. fumigatus* ET3 was found optimal when 24 h-old culture was used as an inoculum (Van Tinh et al. 2017).

2.6 Downstream Processing

Likewise, downstream processing is an integral part of any product development process, since the final costs of the product largely depend on the costs incurred in extraction and purification steps. Downstream processing plays an essential step in the separation of enzymes on the commercial scale. Downstream processing aims mainly to minimize the number of unit operations involved, thus reducing overall process and validation costs, and accordingly simplifying ease and economy of process automation. The complexity of downstream processes is determined by the required product purity and applications. Downstream processing, involving recovery and formulation, incurs 70% of the overall production costs of enzyme due to the complexity of the system and the need to maintain its biological activity.

Separation and purification technologies for phytase, employing a chromatographic process, have evolved slowly as compared to production phase. Most of these approaches were employed for analytical purposes, especially for biochemical, molecular, and structural characterization. Phytase is susceptible toward inactivation; therefore, in order to enhance their stability, phytases are often formulated as solid-state proteins produced by spray drying, lyophilization, or granulation. Dry formulation greatly reduces the likelihood of chemically and biologically mediated inactivation. Thus, there is a growing interest for fast and economic processes, which will stimulate research to unlock new insights in phytase down streaming technology. Various stages of after-fermentation processing include separation, purification, and packaging of the product. Conventional procedures, including pretreatment, precipitation or chromatographic methods, and salt precipitation, are currently employed for phytase purification (Bhavsar and Khire 2014; Bhavsar et al. 2012). These traditional approaches are currently employed due to lack of alternative methods (Ashok et al. 2017). Due to the high commercial potential of phytase, several methods have currently been applied to obtain a highly active phytase suit-

able for industrial applications. Several traditional purification processes have been employed to purify phytase from microbial sources. One of them is the traditional multi-step procedure involving salt precipitation and column chromatography.

2.6.1 Pretreatment and Concentration

In phytase production process, several concentration and purification steps are required to reach the final end step quality product. Certain pretreatments are required because phytases could be produced intracellularly or extracellularly. Depending on the location of the enzyme, various permeabilization treatments including organic solvents, enzymes, detergents, and physical methods are used (Bindu et al. 1998). Solid liquid separation techniques, such as centrifugation and decantation, are usually used for extracellular phytase separation. The culture filtrate is eventually concentrated by salt precipitation, acetone precipitation, and ultrafiltration (Bhavsar and Khire 2014).

2.6.2 Chromatographic Process

Further purification of phytases includes gel filtration, ion exchange chromatography, affinity chromatography, and hydrophobic interaction. The recovery and purification of phytase have been achieved by several steps using different techniques, such as ultrafiltration, diafiltration, ion exchange, gel filtration, and hydrophobic interaction (Konietzny et al. 1995). An extracellular phytase from *A. niger* 11T53A9 was purified about 51-folds by ammonium sulfate precipitation, ion chromatography, and gel filtration (Greiner et al. 2009).

2.6.3 Liquid–Liquid Extraction

Single step aqueous two-phase extraction (ATPE) during downstream processing of phytase produced in SSF has resulted in higher phytase recovery (98.5%) within a short time (3 h) with improved thermostability properties. The ATPE method, therefore, seems to be an interesting alternative for simultaneous partitioning and purification of phytase (Bhavsar et al. 2012). This phytase purification using liquid–liquid extraction is likely to be beneficial in the poultry feed industry. The partition and recovery behavior of phytase, produced by solid-state cultivation utilizing citrus pulp as substrate, was also determined in an ATPE-based process composed of PEG–citrate (Neves et al. 2012). The results suggested that PEG-citrate-ATPE process is another interesting and efficient alternative to the traditional chromatographic methods.

2.6.4 *Immobilization*

Immobilization has been used as a technique in separation and purification of industrial enzymes. *A. niger* phytases were immobilized on natural supports, such as allophone. The residual activity of immobilized phytase on allophanic and montmorillonite nanoclay supports was higher under acidic conditions, and led to a higher thermal stability and resistance to proteolysis (Menezes-Blackburn et al. 2011).

Considering the increased use and demand of phytase, more efforts are needed to produce phytase in a cost effective process accompanied with fast and economic upstream and downstream processing. Phytase production has also been studied under SmF and SSF; and previous studies revealed that enzymatic production under SSF has several advantages in comparison to SmF (Bhavsar et al. 2011; Neira-Vielma et al. 2018). Development of a viable process for phytase recovery and purification with techno-economic feasibility is necessary due to limitations encountered with many of the present methods.

2.7 Formulation

Phytases can be applied into animal feed as dry granules or in a liquid form. The types of formulation chosen depend on the operational conditions used at an animal feed mill. A dry granulated phytase formulation may be added to the mixer before pelleting process. Due to high temperature conditions during mixing and pelleting process, special coated granulated formulation is required to preserve phytase activity. There are many types of granulation technologies available including, but not limited to, pneumatic dry granulation, reverse wet granulation, steam granulation, moisture-activated dry granulation, thermal adhesion granulation, freeze granulation, and foamed binder or foam granulation (Shanmugam 2015). For liquid phytase formulation, phytase is normally added after pelleting process to avoid heat inactivation during pelleting process.

Previous studies reported that phytase from *Shizophyllum* sp. showed more stability in solid formulations containing high fiber levels than the concentrated liquid product at room temperature, both without any additive supplementation (Salmon et al. 2011). Liquid mannitol-containing formulation (1%, w/v) retained 89.83% of phytase activity after 60 days of storage, while polyethylene glycol addition (1%, w/v) to the liquid formulation also retained approximately 90% of phytase activity after 60 days. According to EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP 2017), powder formulation of Natuphos® E 5000 G and 10,000 G containing phytase concentrate, magnesium sulfate (~2%), wheat bran (97%), and vegetable oil (~1%, soybean), and granular formulations containing phytase concentrate (1.5–2.7%), starch (82%), polyvinylalcohol (1.4%), gum arabic (3%), wax-based coating agent (5.0%), and water (up to 100%) are safe for use as feed additives for avian and porcine species.

2.8 Applications of Phytase Enzymes

Phytase enzymes play a crucial role in a wide range of applications, such as human food and nutrition, animal feed and nutrition, and environmental concerns regarding phosphorus pollution. The application of phytase is of vital importance in these areas because phytase works to liberate phytic acid-entrapped phosphorus, an essential element for all living organisms, in major food sources such as soy, corn, wheat, and rice. Accordingly, liberated phosphorus will be biologically available for the consuming organisms.

2.8.1 Food and Feed Industries

Among different nutritional sources for human and animals, cereals such as wheat, maize, rice, and soy still remain primary food sources in the world with 54% contribution of total food consumption in the developing countries (Kearney 2010). Cereal products are important sources of energy, carbohydrates, proteins and fiber, with diverse micronutrients such as vitamin E, some of the B vitamins, magnesium, and zinc, and a range of bioactive ingredients. Therefore, there is a growing interest toward potential health benefits provided by such substances (McKevith 2004).

However, the complete nutrition offered by cereals is unable to be harvested by our monogastric digestion systems. This is due to the presence of phytic acid, an antinutritional factor which needs to be broken down by phytase enzyme to liberate the bound phosphorus. Phytic acid or phytate is the major storage form of phosphate and inositol in plants where 20% of phosphorus is stored as phytate in roots and around 80% is stored in seeds. It can form complexes with proteins, amino acids, and several divalent cations of high nutritional importance, such as Ca^{2+}, Mg^{2+}, Zn^{2+}, Cu^{2+}, Fe^{2+}, and Mn^{2+} (Haefner et al. 2005).

Phytic acid inhibits iron absorption, giving rise to high prevalence of iron deficiency in infants from developing countries, women of childbearing age and vegetarians. Iron deficiency is among the major risk factors for disability and death affecting an estimated 2 billion people around the globe (Zimmermann and Hurrell 2007). Phytase used in food processing and manufacturing industries is very helpful in preventing such nutritional deficiencies. For instance, high phytic acid contents of corn necessitate the action of phytase during corn wet milling process to produce improved products. On the other hand, phytase is considered as a potential savior in bread making sector to produce better nutritional breads with improved texture and faster production times. Additionally, with phytase application can aid in the extraction of plant-based proteins, such as soy protein as an alternative source for the ever-increasing protein worldwide demand. Phytases are also used in animal feed industries, where they afford better digestibility and bioavailability of nutrients from plant-based feed raw materials and overcome problems of environmental pollution with excreted phosphorus.

2.8.2 Corn Wet Milling

Phytases are used in corn wet milling process to produce corn steep liquor. The presence of phytate in the corn kernels produces corn steep liquors with less desirable qualities. Addition of phytases to this process shortens the overall processing time and produces corn steep liquors that are completely void of phytate (Antrim et al. 1998). Żyta (1992) reported that phytase application improved the separation of germ and starch from fiber, reduced corn steeping time, and increased starch and gluten yields. Different fermentation industries for the production of antibiotics, enzymes, amino acids, and high energy animal feed ingredients in liquid forms generally favors the use of corn steep liquors without phytate contamination (Dahiya 2016).

2.8.3 Bread Making

Mankind has been consuming bread for ages and it has become an inseparable part of human nutrition around the globe. The ability of bread to survive the test of time is owed to its adaptability to produce, ease of making, and certainly the wholesome nutrition attributed by cereal grain meals used. Among the main cereals used in bread making are wheat, rye, oats, and barley, and they cater a wide range of important nutrients such as carbohydrates, fibers, proteins, lipids, as well as micronutrients such as vitamins and minerals (Dewettinck et al. 2008). Researches clearly suggested that fiber or other bran constituents are not responsible for the inhibition of iron absorption in wheat and rye bread. This inhibition is mainly attributed to the contents of phytate or its degradation products (inositol phosphate; Brune et al. 1992). Owning to high phytic acid contents, bioavailability of minerals from wholemeal bread is low and corresponds directly to its phytate content (Brune et al. 1992). For consumers to reap the most nutritional benefits from bread, phytic acid contained in the cereal grains needs to be hydrolyzed, and this is where the application of phytase is needed.

Greiner and Konietzny (2006) quoted that exogenous phytase application for food processing is similar to optimizing the dephosphorylation of phytate naturally present in raw materials. In fact, the effectiveness was proven to completely degrade phytate in cereal and legume derived foods as well. Furthermore, Türk and Sandberg (1992) demonstrated that addition of phytase from *A. niger* reduced phytate contents to a level which does not interfere with iron absorption at low pH conditions (3.4–3.6). Additionally, they also found that using fermented milk, instead of fresh milk for bread making is better, because fermented milk improved the mineral bioavailability, a trait not found upon using raw milk. Żyta (1992) recommended that a phytase additive in bread making needs to be safe, highly active, and independent of Ca^{2+} concentration, as it interferes with phytate degradation. The optimal pH and temperature conditions were pH 4.5–5.0 and 30 °C, respectively. Haros et al. (2001)

studied the effect of fungal phytase addition in bread making and produced concluded findings:

(i) Doughs added with fungal phytases required shorter fermentation periods rather than doughs without exogenous enzyme addition; therefore, fermentation step was reduced with phytases.
(ii) Desired bread texture parameters, such as softer bread crumbs, reduced gumminess, and chewiness, were obtained with phytase supplementation.
(iii) Phytases cause the activation of endogenous alpha amylase enzyme which breaks down starch into simple sugars and makes it easier for yeast fermentation.

2.8.4 Synthesis of Lower Inositol Phosphates

Phytases are also beneficial in improving several biochemical processes occurring within animal and plant cells. The phosphoric esters of myoinositol (mono-, bis-, tris-, and tetrakis-phosphates) play a crucial role in transmembrane signaling processes as well as in calcium mobilization from intracellular store in animals and plant tissues (Haefner et al. 2005). Degradation of phytic acid occurs by sequential hydrolysis in reversed steps, which starts from inositol hexaphosphate (IP6) and finally hydrolyzed to myoinositol (IP1). This hydrolysis reaction yields products, such as less phosphorylated myoinositol derivatives, inorganic phosphates, and liberated minerals, which were bound to it, such as calcium, magnesium, potassium, zinc, and copper ions (Liu et al. 1998). Table 2.4 summarizes the functionality for each degradation product.

Table 2.4 Summary of the functionality for each degradation product

Inositol phosphates	Functionality
IP6	Iron transporter and important for neuronal activities in animal cell Stores phosphate acts as an antinutritional factor and antioxidant in plant seeds
IP5	Involve in binding of oxygen to hemoglobin in red blood cells of humans and animals
IP4	Act synergically with IP3 by regulating intracellular communication and synergistic function as an intracellular calcium level controller
IP3	Rephosphorylated to IP4 or catobolized to IP2 activating release of calcium ions from intracellular form
IP2	Activating release of calcium ions from intracellular form.
IP inositol monophosphate	Hydrolyzed to form free myoinositol phosphate, the precursor for the inositol phospholipid, a second messenger in the inter cell signaling system
Myo-inositol	Primary form of nutritional and metabolite function when bound covalently to phospholipids as phosphatidylinositol

Żyta (1992) found that research interest in this field influenced the need for various inositol phosphate preparations. Moreover, this was also influenced by the fact that chemical synthesis of these products was difficult, while enzymatic synthesis showed the advantage of high stereospecificity and the application of mild reaction conditions. The use of phytase derived from *Saccharomyces cerevisiae* has been shown to be very effective in producing different inositol phosphate species, such as D-myoinositol 1,2,6-trisphosphate, D-myoinositol 1,2,5-trisphosphate, L-myoinositol 1,3,4-trisphosphate, and myoinositol 1,2,3-trisphosphate. On the other hand, phytases isolated from *A. niger* efficiently hydrolyzed IP6 to all lower phosphorylated derivatives from IP5 to IP2 (Haefner et al. 2005).

2.8.5 Production of Plant Protein Isolates

The main protein source of human and animal diets is always derived from animals. Fish, meat, eggs, and dairy sources of protein must not be relied completely to feed the high protein demand. The need for an alternative plant-based protein source is necessary to cater the high nutritional needs of increased world population. Plant-based protein sources are less accessible by the digestive enzymes due to the binding of phytate to proteins at extreme pH ranges (Wang 2008). Hence, there is an increased demand to produce phytate-reduced protein isolates as an alternative protein source. Phytate-reduced protein isolates were suggested as suitable protein sources for infant formula and are also regarded as functional additives in food products due to their good foaming, emulsifying, and gelling properties (Dahiya 2016).

Fredrikson et al. (2001) used exogenous phytases to isolate pea protein and managed to completely degrade phytate into hexa, penta, tetra, and triphosphates within 1 h of incubation. Such protein isolates were suggested to be used in infant formula, since they were able to reduce flatulence and improved mineral bioavailability. Żyta (1992) achieved almost complete dephosphorylation of protein isolates from soybean within few hours of using intracellular acid phosphatase rich in phytase activities derived from *A. niger*. Wan et al. (2015) explored the use of plant proteins, especially soy, corn, and wheat proteins, as various delivery platforms, such as micro- and nanoparticles, fibers, films, and hydrogels for bioactive ingredients.

Apart from providing nutrition, plant-based proteins must also possess certain functional properties such as solubility, binding properties, surfactant properties, and viscogenic texturizing characteristics. For example, pea proteins which are now industrially produced in Europe are increasingly used in food for their good emulsifying and foaming properties (Chéreau et al. 2016).

2.8.6 Phytases in Animal Feed

The potential application of phytase enzymes in animal feed industry has garnered attention from the feed manufacturers as a cost-effective and efficient way to improve animal performance (Kumar et al. 2017; Yadav et al. 2017). In 1991, the first commercial phytase products were introduced into feed market as feed additives for swine, poultry, and aquaculture feeding sectors (Greiner and Konietzny 2006). The wide application of phytase in various farming practices is mainly attributed to its high market value, which is estimated to surpass USD 300 million, with an annual increase of 10% (Chen et al. 2015). Phytase in animal feed is welcomed greatly because it not only helps to degrade phytate for better bioavailability, digestion, and absorption of nutrients from the feed, but it also prevents excessive phosphorus animal excretion and its environmental hazards. Phytase can therefore play a multiple role in animal feed, whereby it could improve animal performance, reduce the need for additional phosphorus in animal feed, and formulate eco-friendly feeds, which reduce the excretion of excessive phosphorus to the environment.

Haefner et al. (2005) reported that phosphorus excretion could be almost reduced by about 50% with phytase application, which is considered as a significant contribution toward environmental protection. Furthermore, addition of adequate amounts of phytase cuts the need to provide phosphorus supplements in diets of monogastric animals. Experimental trials in both laboratories and fields have continuously shown that 1 g of inorganic phosphorus supplementation can be replaced by 500–1000 units of phytase, with 30–50% reduction in total phosphorus excretion (Yao et al. 2012). Alongside with these benefits, phytase is also believed to contribute toward disease prevention in animals (Romano and Kumar 2018). Phytases can also be used as a good pretreatment for the feed raw materials. Optimized phytase activity could be achieved by limiting the amount of phosphorus, calcium, and certain organic acids in animal diets as these compounds may act synergistically to affect feed digestibility (Romano and Kumar 2018).

2.8.7 Phytases in Broiler Diets

Maximal absorption of nutrients is highly important for broilers from feed sources, as they need to achieve optimal growth in a short period of time. Thus, presence of antinutritional factors in broiler diets originating from plant-based ingredients becomes a hindrance for the broiler growth. Phytases have been successfully applied in broiler feeds for their various effects on growth and performance. Many researches have been conducted to discover the multiple potentials of phytases in broiler diets. Scholey et al. (2018) studied the effects of phytase supplementation to broiler diets low in inorganic phosphorus. Their results supported the positive effect of phytases in growth performance and bone mineralization of broilers. However, 1000 phytase

units were the minimum recommended dose required to satisfy the complete need of phosphorus in broiler diets. Bradbury et al. (2017) highlighted the importance of replacing limestone as a calcium source for broiler diets with High Soluble Calcium source (HSC). High inclusion of limestone could cause hazardous effects to the broiler in terms of phosphorus and amino acid digestibility and also increases the pH of gastric juice. Replacing limestone with HSC together with phytase supplementation has the potential to be an excellent tool to improve broiler body weight gain, feed intake, and bone mineralization.

2.8.8 Phytases in Swine Diets

Supplementation of 500 phytase units/kg and above, effectively hydrolyzed phytate in low-phosphorus corn-soybean diets for pigs, while a huge dose of 20,000 phytase units/kg hydrolyzed almost all the phytate and further improved mineral and protein utilization and performance in terms of weight gain, feed intake, feed efficiency, bone breaking strength, and bone weight (Zeng et al. 2014). Yitbarek et al. (2017) studied the effect of phytase supplementation on greenhouse gas emissions from soils after manure application. They found out that the effect of phytase in swine diet does not only influence or has little influence on the emission of greenhouse gasses, but the excretion of P and N in swine manure was also greatly reduced. Appropriate dietary calcium and phosphorous concentrations are essential for nursery pig performance; however, too much Ca in young swine diets decreases its performance and bone ash contents. Addition of standardized digestible phytases (minimum 0.45%) to the swine diet could improve the average daily gain and feed intake (Wu et al. 2017).

2.8.9 Phytases in Aquaculture Feed

Application of fishmeal to feed aquaculture industry is not a sustainable approach because fishmeal production is limited, and it is estimated that the demand itself will soon exceed its yearly production. Hence, the solution would be to replace fishmeal with alternative ingredients derived from crops, such as soybeans, wheat, corn, or rice (Hardy 2010). Replacing fishmeal with plant protein comes with its own challenge as well. Plant proteins are rich in phytic acid. Like humans, aquatic animals are not able to hydrolyze this compound too. In pisciculture sites, those phytic acid compounds would stay in ponds or rivers into which waste water is discharged, contributing to eutrophication. Compared to fish-based protein meal, plant protein usually contains more indigestible organic matter such as insoluble carbohydrates and fiber and some mineral compounds such as phosphorus which have limited uptake in fish leading to higher levels of fish excretion and waste (Naylor et al. 2009).

Since plant proteins are increasingly used to replace fish meal for aquaculture feed production, there is a niche area for phytase to greatly improve the nutrient

bioavailability of those plant proteins. In aquaculture, the use of phytase to improve phosphorus utilization has been already in use (Castillo and Gatlin III 2015). Many research works have focused on the effect of phytase in aquaculture feed and have produced sufficient evidence which demonstrate the effectiveness of phytase when added to aquaculture feed.

It was observed that adding phytase into the Nile tilapia diet improved digestibility of several nutrients including protein, carbohydrates, energy, ash, phosphorus, and calcium. Moreover, combination of xylanase and phytase resulted in synergistic effect on the Nile tilapia growth as well (Maas et al. 2018). In another study by Sugiura et al. (2001), phytase supplementation to rainbow trout diet also show to increase the absorption of phosphorus, protein, ash, calcium, magnesium, copper, iron, strontium, and zinc. Excretion of phosphorus was also reduced to an extent of 95–98% compared with phosphorus excretion by fish consuming feeds without phytase. Apart from fish diets, effects of phytase supplementation were evaluated on shrimp diets as well. Phytase supplementation was able to improve nutrient retention for compounds, such as Cu, P, proteins, and some amino acids in Pacific white shrimps (*Litpenaeus vannamei*), while other improvements such as weight gain and feed conversion ratio were not achieved (Qiu and Davis 2017). Another study with tra catfish (*Pangasianodan hypopthalmus*), phytase supplementation improved growth performances, feed, and phosphorus utilization. Moreover, the supplementation was able to eliminate the need of dicalcium phosphate or any other additional phosphorus sources in catfish feed and reduced the phosphorus excretion into environment (Hung et al. 2015).

2.8.10 Phytases in Ruminant Feeds

In spite of the wide application of phytases in animal feed, little information is available about the potential advantages of exogenous supplementation of this enzyme to ruminant feeds. Many reports showed the insignificant effect of phytase addition to dairy cows in terms of phosphorus digestibility and milk yield (Humer and Zebeli 2015; Winter et al. 2015). However, other studies reported that even though phytate could be digested by ruminants, it is believed that more than 60% of the P consumed by dairy cattle can be excreted in feces with a potential to cause environmental pollution. Therefore, use of phytase was suggested as feed supplement in cattle farming to overcome this problem (Kebreab et al. 2013).

2.9 Recent Novel Applications of Phytases

Recently, researchers are looking to explore more on the various capabilities of phytases, and producing new data which shows that phytase has more layers of functionality. Soni et al. (2015) utilized the advantage of the highly glycosylated characteristic of phytase and used it for drug delivery applications. It was found that

self-assembled phytase enzyme nanospheres possess antitumour properties and these can be further enhanced by loading the phytase nanosphere with curcumin, an anticancer drug. In agricultural sectors, the increasing demand for fruits, vegetables, and grains pressurizes farmers to increase crop productivities. Hence, they are urged to use pesticides extensively to produce more crops with less pest damage. Organophosphorus is one such pesticide used in agriculture, but unfortunately it can't be easily eliminated by washing and rinsing the crop with water and leads to its bioaccumulation in the food chain (Vendan 2016). Residues of this pesticide left in the crop cause harmful effects on the nervous system of exposed animals and humans (Mileson et al. 1998). Needless to say, it is crucially important to detoxify the crops exposed to pesticides. Shah et al. (2017) discovered an interesting novel potential of phytase as an organophosphate detoxifying agent in agriculture crops. In their research, phytase produced from *A. niger* NCIM 563 can degrade 72% of organophosphate under normal conditions, pH 7.0, and 35 °C on green chillies.

2.10 Conclusions and Future Trends

At present, phytases are widely used in many feed industries and are mainly produced using microbial sources. The current annual market volume exceeds USD 350 million. This number is expected to increase in parallel to the rapid growth of poultry, swine, and aquaculture farming. In addition, the increased awareness about the negative environmental impacts of phosphorus released is another driving force for the increased usage of phytases in animal feed. Based on their application in animal feed industries, commercial phytases are different in terms of pH, temperature profiles, and overall enzyme properties. Nowadays, more research is focusing on protein engineering to improve enzyme stability to withstand higher temperatures applied during feed production processes. Furthermore, with the increased available data of phytase genes and the ease of construction of new recombinant microbes, many new overproducer strains are now available in different fermentation industries. Another trend is the application of heterologous expression systems to integrate the thermostable phytase gene in plants. This is going to reduce the cost of phytase production processes and the use of plants as integrated biofactory of both nutrients and feed enzymes.

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Chapter 3
Fungal Probiotics: Opportunity, Challenge, and Prospects

Abhijit Banik, Suman Kumar Halder, Chandradipa Ghosh, and Keshab Chandra Mondal

3.1 Introduction

The word 'probiotics' is derived from its Greek meaning 'for life,' first coined by Lilley and Stillwell in 1965 (Fuller 1992). Probiotics are actually a therapeutic preparation containing living forms of nonpathogenic microorganisms that will ultimately benefit the host body by balancing nutritional content and the immunological network, and also by preventing colonization of pathogenic microorganisms (FAO 2006; Martín et al. 2005). Heterogeneous groups of the beneficial fungal community are now showing a new horizon in the probiotic market (Agheyisi 2014; Yadav et al. 2017b). The diverse biological importance of fungal probiotics has attracted the attention of researchers and industries concerning its commercial prospects. Fungi (fungus, singular; fungi, plural; Latin word 'fungus' means mushroom) are basically nonphotosynthetic, spore-bearing heterotrophic eukaryotic organisms that reproduce both sexually (by spore formation) and asexually (by budding). When living freely in water and soil, fungi also form a symbiotic relationship with animals and plants (Dube 2013). Among the diverse family of fungi, yeasts are unicellular microorganisms belonging to the phylum Ascomycota under the class Saccharomycota. Yeasts are widely distributed in several ecological niches such as the normal human gastrointestinal (GI) flora, on plants, in water, in airborne particles, and also in various traditional fermented and nonfermented food products (Rima et al. 2012). Molds (moulds) are another important group of fungal species that grow in multicellular filaments called hyphae. Both yeasts and molds are of great significance in food processing and fermentation technology (Holzapfel

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2002). From ancient times, the diverse community of fungi has benefitted society in a number of ways.

3.2 Uniqueness of Fungal Probiotics

The unique cellular architecture of fungi makes it a better class of probiotics than commercially available bacterial probiotics because the cell envelope of yeast is composed of two layers: the outer layer consists of mannan (phosphopetidomannan or phospholipomannan) and the inner layer contains chitin and 1,3- and 1,6-β-glucan (Lipke and Ovalle 1998). This structure allows easy transition through the gastrointestinal environment. Several genera of fungi also grow optimally in varied temperatures. Their antagonistic property toward numerous microorganisms inhibits the growth of pathogenic bacteria in the gut. Several other properties of the fungal community that are directed toward probiotic characteristics are listed here. All these basic characters of the fungal family have fulfilled the potentialities for a probiotic candidate.

3.3 Fungal Genera and Species as Probiotics

A group of fungal genera have been reported as novel candidates in the probiotic family: *Candida humilis*, *Debaryomyces hansenii*, *Debaryomyces occidentalis*, *Kluyveromyces lactis*, *Kluyveromyces lodderae*, *Kluyveromyces marxiamus*, *Saccharomyces cerevisiae* var. *boulardii*, *Pichia kluyveri*, *Issatchenkia orientalis*, *Pichia kudriavzevii*, *Candida tropicalis*, *Meyerozyma caribbica*, *Candida saitoana*, *Candida pintolopesii*, *Cryptococcus albidus*, and *Torulaspora delbrueckii* (Kumura et al. 2004; Maccaferri et al. 2012; Martins et al. 2005; Ochangco et al. 2016; Smith et al. 2016; Smith et al. 2014; Srinivas et al. 2017; Puppala et al. 2018; Cho et al. 2018; Amorim et al. 2018; Fadda et al. 2017; El-Baz et al. 2018). The best studied yeast is *Saccharomyces boulardii*.

3.4 Advantages of Fungal Probiotics

The most commercially available yeast strain is *Saccharomyces boulardii*. *S. boulardii* is a unique, nonpathogenic, tropical yeast (fungus) that can support health in a number of ways (Sharma and Saharan 2018) (Fig. 3.1). Current reports suggest that use of the yeast strains, singly or in combination with other probiotics, supports GI function by increasing populations of good bacteria with concomitant decrease in pathogenic organisms by means of competition for space and food (Sartor 2004). In the 1920s the French scientist Henri Boulard isolated the yeast *S. boulardii* strain

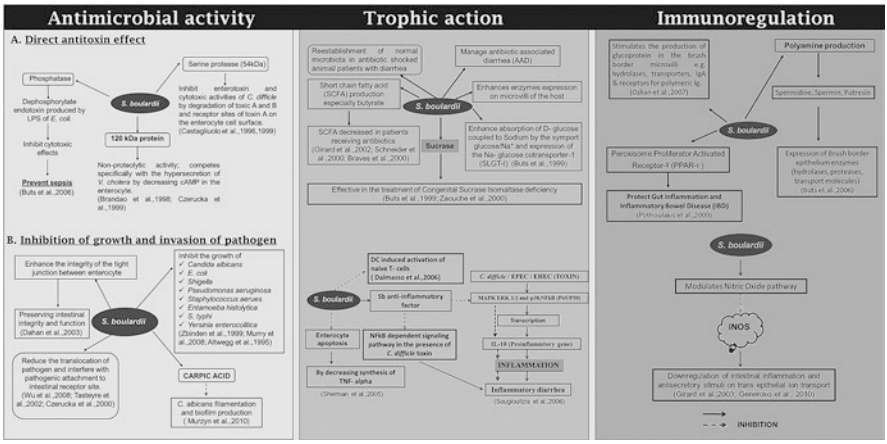

Fig. 3.1 Several postulated health benefits of *Saccharomyces boulardii*

for the first time from lychee and mangosteen, and noticed that chewing the skin of these fruits helps to control cholera of the natives of Southeast Asia (McFarland 2010). In contrast to most other probiotics, which are bacteria based, *S. boulardii* is a yeast probiotic supplement reported as a beneficial microorganism, able to survive in the acid environment of the stomach and colonize the GI tract; this probiotic is reported to encourage the growth of friendly bacteria to maintain gut health, digestive health, and immunity (Kumar et al. 2017; Yadav et al. 2017a).

3.5 Fungal Probiotics in Human Health

Numerous findings suggest that fungal-based probiotics affect the host in several aspects (Table 3.1).

3.5.1 Health Beneficial Effects in the Normal Physiological State

First, production of antitoxic factors against several enteric bacterial toxins such as *Escherichia coli* lipopolysaccharides (LPS), toxins A and B of *Peptoclostridium difficile*, and *Vibrio cholerae* toxins are reported for the yeast strains (Buts and De Keyser 2006). The antimicrobial activity of probiotic yeasts can also help to preserve the occluding junctions of the cells, especially tight junctions, by E-cadherin recycling, thus alleviating colonization by pathogenic bacteria as well as maintaining the integrity of the intestinal epithelium (Bisson et al. 2010; Ooi et al. 2009). By modulating metabolic activity and increasing the production of short-chain fatty

Table 3.1 Summary of major health beneficial effects of fungal probiotics

Action of fungal probiotics	Fungal candidates as probiotic	Beneficial effects on host body	References
Probiotic effects	*Saccharomyces cerevisiae, Kluyveromyces marxianus, Metschnikowia gruessii, Meyerozyma caribbica, Pichia membranifaciens, Candida oleophila*	Colonization, resistance, and inhibition of enteric bacterial pathogens Maintenance of epithelial barrier integrity Antiinflammatory effects Immunomodulation activity Effect on mucosal layer of intestine-trophic effects Clinical effects on different types of diarrhea, e.g., AAD, traveler's diarrhea, HIV-associated diarrhea Eradication of *Helicobacter pylori* infection Reduce blood cholesterol level	Lessard et al. (2009), Amorim et al. (2018), Silva-Aciares et al. (2011), Butler et al. (1991), Qamar et al. (2001), Czerucka et al. (2007), McFarland (2007), Ragon et al. (2008), Smith et al. (2014).
Dietetic effects	*Saccharomyces cerevisiae, Saccharomyces kluyveri, Kluyveromyces lactis, Pichia anomala, Pichia rhodesiensis, Pichia spartinae, Torulaspora delbrueckii, Candida krusei, Pichia pastoris, Schwanniomyces castellii, Rhodotorula gracilis, Pichia membranifaciens*	Biodegradation of phytate Enhance bioavailability of significant ions, e.g., iron, zinc, calcium, magnesium Release of oligosaccharides Production of all B-complex vitamins	Silva-Aciares et al. (2011), Ragon et al. (2008), Lim et al. (2008), Fernández et al. (2015)
Bio-fortification of folate	*Saccharomyces cerevisiae, Saccharomyces pastorianus, Saccharomyces bayanus, Pichia anomala, Kluyveromyces marxianus, Candida glabrata, Debaryomyces hansenii, Debaryomyces vanrijiae*	Prevent neural tube defects in fetal stage of embryo Prevention of megaloblastic anemia Reduce the risks of occurrence of cardiovascular disease, cancers, osteoporosis, and Alzheimer's disease	Hjortmo et al. (2008), Witthuhn et al. (2005), Hjortmo et al. (2008)

(continued)

Table 3.1 (continued)

Action of fungal probiotics	Fungal candidates as probiotic	Beneficial effects on host body	References
Absorption and destruction of mycotoxins	*Saccharomyces cerevisiae, Saccharomyces boulardii, Phaffia rhodozyma, Xanthophyllomyces dendrorhous*	Antitoxic effects Regulates aflatoxin production	Péteri et al. (2007), Sabater-Vilar et al. (2007), Silva et al. (2015)

acids (SCFAs), especially butyrate, the probiotic yeast controls gut microfloral balance and prevents dysbiosis (Swidsinski et al. 2008; Czerucka et al. 2007).

Second, the probiotic yeast *S. boulardii* can affect the intestinal mucosa with the maturation of enterocytes by stimulating the secretion of trophic polyamines such as spermine and spermidine. The increased level of yeast-generated polyamines acts as a signaling molecule through the polyamine transport system (PTS)(Czerucka et al. 2007). Diamine oxidase (DAO), a degradation enzyme, in turn negatively regulates the concentration of polyamines. Experimental evidence also suggests that *S. boulardii* significantly increases the level and activity of brush-border membrane (BBM) enzymes such as sucrase-isomaltase (SI), lactase-phlorizin hydrolase (LPH), maltase-glucoamylase (MGA), α-glucosidase, intestinal alkaline phosphatase (IAP), and amino peptidase N (APN), although the activity of all these enzymes is variable in the apical and basal parts of the BBM. All the aforementioned digestive enzymes have great importance in the breakdown of nutrients and intestinal absorption for the benefit of both yeast and host. If there are any abnormalities in the small intestine or nutrient malabsorption, probiotic strains can provide appropriate beneficial actions. When orally administered to experimental rats, *S. boulardii* causes increased absorption of D-glucose and thus modulates disaccharide activity by the coactivity of sodium glucose symporter. *S. boulardii* is also responsible for the elevated expression of sodium glucose cotransporter-1 (SLGT-1) in the BBM, the ultimate site of the digestion of carbohydrates and absorption of water and nutrients (Buts et al. 1999).

Third, probiotic yeasts also regulate the immune secretory activity of the intestinal epithelium. Yeast cells mainly interact with epithelial cells to trigger both innate and adaptive immune response by recruitment of specialized cells customized for both these responses (Rodrigues et al. 2000). *S. boulardii* especially modulates the secretion of immunoglobulin and secretory IgA in the intestinal lumen, polymeric immunoglobulin receptors, and human membrane proteins, and reduces release of phospholipase A2, an enzyme that acts as a stimulator of proinflammatory lipid mediator (platelet-activating factor and eicasonoids), interleukin (IL)-6, IL-8, tumor necrosis-alpha (TNF-α), and chemokines such as CCL2, CCL20, and CXCL-8 (Buts et al. 1990; Qamar et al. 2001; Ozkan et al. 2007; Badia et al. 2012). Several

food-isolated yeast genera such as *Debaryomyces*, *Kluyveromyces*, and *Metschnikowia* display highly diverse and strain-dependent dendritic cell (DC)-inducing properties. Dendritic cells have fundamental roles in the regulation of adaptive immune response as well as secretion of inflammatory cytokines. For instance, TNF-α and IL-1b are key cytokines for the acute innate inflammatory responses that attract macrophages and neutrophils to the site of action (Smith et al. 2014). In vitro findings proved that *S. boulardii* alters the signaling pathways involved in pro-inflammatory cytokine synthesis. In this way, by modulating the activity of immune cells, yeasts provide a beneficial impact on human health.

Fourth, yeasts have a positive influence on the growth of crypt cells and villus cells by enhancing the height, width, and number of goblet cells (Bontempo et al. 2006). Nonpathogenic fungal strains also interfere with quorum-sensing cross-talk, a cell signaling pathway important for the pathogenic profile and morphogenesis of certain pathogens. Colonization by *Saccharomyces boulardii* modulates the behavior of normal gut flora (Dahan et al. 2003).

Fifth, several probiotic yeast strains, for example, *Debaryomyces castellii*, *Saccharomyces cerevisiae*, *Saccharomyces kluyveri*, *Pichia anomala*, *Pichia spartinae*, *Torulaspora delbrueckii*, *and Kluyveromyces lactis*, showed nutritional effects in the host body by phytase activity and by enhancing the bioavailability of zinc, iron, calcium, and magnesium. Phytic acid (myo-inositol hexakiphosphate), a conserved form of phosphorus, is considered to be a nonnutritional and chelating agent that decreases the availability of proteins and several ions (Ragon et al. 2008; Olstorpe et al. 2009). Phytases, particularly those produced by fungi, cause hydrolytic degradation of phytic acid to free inorganic phosphate and provide lower myo-inositol phosphate esters (Lim et al. 2008; Fernández et al. 2015).

Several studies also showed that probiotic fungal strains such as *Saccharomyces cerevisiae*, *S. pastorianus*, *S. exiguous*, *Metschnikowi lochheadii*, *Debaryomyces hansenii*, *Pichia philogaea*, *P. anomala*, *Candida cleridarum*, *C. glabrata*, and *Kluyveromyces marxianus* also exert nutritional effects by folate bio-fortification. Vitamin B_9 is commonly known as folate, an important cofactor for a carbon transfer reaction in the physiological system. Folate is also beneficial for the synthesis of purine and methionine and interconversion between serine and glycine. Thus, folate aids in cellular replication and growth (Hjortmo et al. 2008). Fungal probiotic members are more active in folate biosynthesis and produce high levels per weight. Folate bio-fortification in turn prevents neural tube defects in the growing fetus, reduces the chance of megaloblastic anemia, and also moderates the risk of cardiovascular disease, cancer, Alzheimer's disease, and osteoporosis. Budding yeasts such as *S. cerevisiae* also reduce the extent of the absorption of mercury (present in water and food matrices) by the intestine (Jadán-Piedra et al. 2017).

Another important positive health benefit of fungal probiotics is the biodegradation and absorption of mycotoxins. Mycotoxins are the secondary metabolites produced by fungi, and among these the fungal genera *Aspergillus*, *Fusarium*, and *Penicillium* are pioneers. Some well-known mycotoxins are aflatoxins, ochratoxins, fumonisins, deoxyniyalenol (DON), zearalenone (ZOA), and trichothecenes.

Contamination of agricultural products, food, and animal feeds by mycotoxins causes various diseases in humans as well as livestock. Degradation of mycotoxins by probiotic strains has been reported, suggesting that degradation of zearalenone leads to conversion into alpha- and beta-zearalenol. *S. cerevisiae* also causes degradation of ochratoxin A, fumonisins B1 and B2, deoxynivalenol, and T2 toxin. By an enzyme-mediated reaction, especially by carboxypeptidases, two fungal strains, *Phaffia rhodozyma* and *Xanthophyllomyces dendrorhous*, can degrade ochratoxin A and convert it into nontoxic ochratoxin-α (Péteri et al. 2007; Sabater-Vilar et al. 2007; Silva et al. 2015).

3.5.2 Health Beneficial Effects During Adverse Physiological States

There is actually a mutualistic relationship between gastrointestinal microbiota and the host body. If this condition is interrupted, dysbiosis occurs, causing severe acute and chronic physiological conditions such as antibiotic-associated diarrhea (AAD), inflammatory bowel disease (IBD) (e.g., Crohn's disease and ulcerative colitis), irritable bowel disease, food allergies, cardiovascular disease, and cancer (e.g., colorectal cancer). Fungal probiotics support health beneficial effects in adverse physiological conditions and also prevent the occurrence of several diseases in humans and animals.

3.5.3 Antiproliferative Effects of Fungal Probiotics

Various in vitro and in vivo studies have shown that candidate fungal probiotic strains can prevent the manifestation of cancer by their antiproliferative effects (Silva et al. 2015; Chen et al. 2009). A considerable amount of research indicates that the probable mechanisms of antiproliferative effects include antitoxin effects against several toxins, antimicrobial and anti-yeast activity, production of bioactive compounds such as oligosaccharides, SCFA, and anti-tumorigenic and anti-carcinogenic compounds, tropic effects on enterocytes, inactivation of carcinogenic compounds, improvements in intestinal barrier function, immunomodulation to boost the host immune system, modulation of physiochemical conditions of the colon environment and antioxidant properties (Ghoneum et al. 2008; Mumy et al. 2008; Weiler and Schmitt 2003; Butler et al. 1991; Foligné et al. 2010; Kogani et al. 2008; Križková et al. 2001). It was reported by different researchers that the probiotic strain *S. boulardii* directly inhibits the activation of extracellular signal regulated protein kinase (ERK1/2) through effects on epidermal growth factor receptor (EGFR) or other RTK signaling pathways (Chen et al. 2006; Chen et al. 2013).

Probiotic yeasts also elevate the expression of the pro-apoptotic protein Bax as well as inhibit the expression of the antiapoptotic protein Bcl2, and by counterbalancing these two proteins, it causes induction of apoptosis in cancerous cells (Ghoneum et al. 2008; Kroemer and Reed 2000). Another probiotic candidate, the yeast *Kluyveromyces marxianus* (AS41) isolated from dairy products, has also shown anticancer activity by secretion of metabolites through downregulating the expression of Bcl2 and upregulating the expression of BAD, CASP9, CASP8, and CASP3, thus inducing the apoptosis of epithelial cancer cells (AGS) (Saber et al. 2017). Administration of fungal probiotics to cancerous mice also increases the recruitment of macrophages on tumor cells, and in turn these induce an apoptotic pathway over the neoplastic cells without affecting normal cells. Thus, fungal probiotics should be beneficial for future application in cancer therapy.

3.5.4 Antiinflammatory Effects of Fungal Probiotics

Fungal probiotics decrease the chances of inflammation by promoting beneficial effects on mucosal epithelial cells through modulation of the host immune system involved in the process of inflammation. Fungal probiotics can modulate the expression of several regulatory inflammatory genes such as cyclooxygenase-2 (COX-2) and NF-κB as well as counterbalance pro-/antiinflammatory cytokines [e.g., IL-6, IL-8, IL-10, IL-1α, IL-1β, interferon-gamma (IFN-γ), TNF-α] and chemokines levels and also produce antiinflammatory factors against various harmful toxins (Dahan et al. 2003; Nurmi et al. 2005; Mumy et al. 2008). From in vitro studies, it has been proved that probiotic yeasts, especially *S. cerevisiae* var. *boulardii*, interfere with the host cell signaling cascade responsible for the elevation of pro-inflammatory responses during infection caused by pathogenic bacteria. The exact mechanism of the antiinflammatory effect shown by *S. cerevisiae* is based on blocking of pro-inflammatory mediators like NF-κB, MAPK (p38 and JNK), and AP-1 in the intestine and also stimulates the expression of peroxisome proliferator-activated receptor-gamma (PPAR-γ) in human colonocytes. In such ways, probiotic yeast can downregulate the expression of IL-6, IL-8, IL-1α, IL-1β, IFN-γ, and TNF-α and upregulate the expression of IL-10 (Lee et al. 2005). Furthermore, probiotic administration in a dose-dependent manner causes restoration of intestinal permeability and protects against occurrence of chronic inflammation and ulcerative colitis (Tiago et al. 2015). In addition, *S. cerevisiae* var. *boulardii* showed an inhibitory effect on inducible nitric oxide synthase (iNOS) activity and the production of nitric oxide (NO). Inhibition of NO reduces the chances of inflammation and thus should be helpful in the treatment of IBD (Girard et al. 2005).

3.5.5 *Health Beneficial Effects of Fungal Probiotics in Clinical Conditions*

3.5.5.1 Antibiotic-Associated Diarrhea (AAD)

Oral administration of antibiotics sometimes causes disturbances in the normal microflora of the host, and colonization by some opportunistic pathogens, mainly *Clostridium difficile*, leads to the adverse inflammatory condition in the intestinal mucosa termed antibiotic-associated diarrhea (AAD) (Katz 2006). Several other pathogenic microorganisms related to the occurrence of AAD are *Staphylococcus aureus*, *Clostridium perfringens*, *Klebsiella oxytoca*, *Candida* spp., *Escherichia coli*, and species of *Salmonella*. It has been reported from randomized clinical trials that fungal probiotics, especially *Saccharomyces boulardii*, reduce complications related to AAD in adults and children after administration in a dose-dependent manner (Kotowska et al. 2005; Surawicz et al. 2000).

3.5.5.2 Traveller's Diarrhea (TD)

In developing countries, traveller's diarrhea is a commonly occurring public health problem during traveling that is associated with nausea, abdominal cramps, fever, and bloating. Pathogenic bacteria such as enterotoxigenic *Escherichia coli* (ETEC), *Shigella*, and *Salmonella* may contribute to 80% of TD cases. *Norovirus* and *Giardia* are also responsible for 10% of TD cases. The efficacy of *S. boulardii* was assessed for 1016 travelers visiting different countries (Kollaritsch et al. 1993).The occurrence of TD was 40%, 34%, and 29% in patients receiving placebo, *S. boulardii* 250 mg per day ($p = 0.019$), and *S. boulardii* 1 g per day ($p < 0.005$), respectively. Meta-analysis of a fungal probiotic was also done in 12 different studies for the reduction of TD cases, and these studies proved that the combination of the two probiotics, that is, *S. boulardii* and *Bifidobacterium bifidum* with *Lactobacillus acidophilus,* significantly prevents traveller's diarrhea (Sanders and Tribble 2001).

3.5.5.3 *Helicobacter pylori*-Related Diarrhea

Chronic levels of gastritis and peptic ulcer in adults and children are commonly caused by the colonization of *Helicobacter pylori* on the gastric lining of the host mucosa. Infection from *H. pylori* is sometimes a risk factor for gastric malignancy in adults. Several studies reported that a fungal probiotic reduces common diarrhea-related symptoms, epigastric pain, taste disturbances, and the nausea and side effects caused by medication. Although *S. boulardii* had no significant effect on the full eradication of *H. pylori*, its oral supplementation alleviated the chances of *H. pylori* infection in adults (Cremonini et al. 2002; Hurduc et al. 2009; Gotteland et al. 2005).

3.5.5.4 Inflammatory Bowel Disease (IBD)

Inflammatory bowel disease is generally characterized by chronic inflammation of the mucosal epithelial cell surface of the intestine. Crohn's disease and ulcerative colitis are the two types of IBD found in humans. Disintegration of the mucosa in the small intestine and colon affects the habitat of normal microflora, ultimately causing diarrhea, abdominal pain, and bleeding through the GI tract. A fungal probiotic candidate reduces the occurrence of dysbiosis by maintaining mucosal integrity and thus preventing the chances of IBD. Several randomized, double-blind, controlled studies evaluated that administration of a yeast probiotic, especially *S. boulardii* in a dose- and time-dependent manner, combined with conventional therapy, decreases the frequency of bowel movements and the probability of the translocation of bacteria in comparison with a placebo group, without showing any side effects on the host body during the trial period. In addition, a placebo-controlled study showed that treatment with yeast probiotic plus conventional therapy results in clinical remission for 68% of ulcerative colitis patients and that improvement will also occur in intestinal permeability with a reduced lactulose/mannitol ratio in patients with Cohn's disease. Hence, a fungal probiotic could be treated as a safe therapy for the prevention of IBD (Maupas et al. 1983; Plein and Hotz 1993; Guslandi et al. 2000, 2003; Vilela et al. 2008).

3.5.5.5 HIV-Associated Diarrhea

Diarrhea is the most common pathological consequence among patients infected with human immunodeficiency virus (HIV). About 60% of patients undergoing antiretroviral therapy (ART) reported diarrheal symptoms, and it was suggested that 19% of these conditions result from the side effects of ART itself. Randomized, controlled, double-blind studies were conducted for the evaluation of the efficacy of the fungal probiotic *S. boulardii* var. *cerevisiae* on acquired immunodeficiency syndrome (AIDS) patients suffering chronic diarrhea. *S. boulardii* var. *cerevisiae* showed positive results, clearing all the diarrheal symptoms in 61% of cases after treatment for 1 week at a specific dose. All the clinical trials regarding the efficacy of this fungal candidate in the treatment of diarrhea indicate it could be a good therapy for the future prevention of HIV-associated diarrhea.

3.5.5.6 Acute Gastroenteritis

Acute gastroenteritis (AGE) contributes from 5% to 10% of the total deaths and is the second cause of death in the most susceptible of the world's population in the under-five age group, caused mainly by bacteria, virus, or parasite. Dehydration, abdominal pain, cramps, and nausea and vomiting accompanied by fever and anorexia, are the main symptoms of gastroenteritis in acute conditions. Rotavirus is the major contributor for AGE in the death profile for children worldwide. A recent

meta-analysis revealed that the fungal-based probiotic candidate *S. boulardii* (at 250 mg twice per day, up to 5 days), significantly decreased the duration of AGE ($p < 0.0001$) in comparison with a control group, without any ill effects in pediatric patients. Thus, the fungal probiotic could be the sole effective therapy for the future management of AGE with proper treatment guidelines (Padayachee et al. 2018).

3.5.5.7 Giardiasis

Giardiasis is the most common parasitic disease. Caused by cysts of *Giardia lamblia*, giardiasis is characterized by chronic diarrhea, pain in the abdomen, and severe weight loss and actually results from consuming contaminated water or food. In some cases, vomiting, bloody stool, and fever may also occur. The effectiveness of the commercial fungal probiotic, *S. boulardii*, in the treatment of giardiasis was assessed by several placebo-controlled double-blind studies. These studies confirmed that *S. boulardii* in combination with metronidazole (750 mg) clears all the symptoms related to giardiasis, and also no giardiasis-causing cysts were found in the treated group. These results indicate that *S. boulardii* has the ability to treat giardiasis with metronidazole and should be used as a future medication (Kelesidis and Pothoulakis 2012; Besirbellioglu et al. 2006; Mansour-Ghanaei et al. 2003).

3.5.5.8 Vulvovaginal Candidiasis

Vulvovaginal candidiasis (VVC), commonly called 'vaginal yeast infection,' is caused mainly by *Candida albicans* affecting women. Vulval itching, vulval soreness, irritation accompanied by dyspareunia, dysuria, and abnormal vaginal secretion are the main VVC-related complications. In vitro studies clearly showed that regular intravaginal administration of the yeast probiotic *S. cerevisiae* significantly inhibits expression of several fungal components (e.g., secretory aspartyl proteinases) responsible for the occurrence of VVC at the vaginal level by modulating the inflammatory profile of the host body. Further clinical trials in humans are needed to prove *S. cerevisiae* is a good therapeutic agent for vaginal candidiasis (Falagas et al. 2006).

3.5.5.9 Acne Vulgaris

Acne vulgaris, or acne, is a chronic inflammatory skin complication that impacts public health and requires a long time for recovery. The effectiveness and tolerance of *Saccharomyces cerevisiae* for 139 patients with various form of acne was evaluated in a randomized double-blind controlled study. This study proved that *S. cerevisiae* significantly reduces acne in 80% of individuals receiving a live yeast suspension, in comparison with a placebo group (Weber et al. 1989). *S. cerevisiae* can be exploited as a future nonconventional therapeutic agent.

3.6 Challenges Regarding the Use of Fungal Probiotics

The past decades have witnessed the significance of fungal probiotics in the treatment of an array of disease conditions. However, the main problem regarding the use of fungal-based probiotics is the dosage and viability of the supplied organisms. The lack of proper standardized protocols and bio-safety issues are two major hindrances in the application of fungal probiotics as therapeutics. Because appropriate data are scarce, application of fungal probiotics in industry does not fulfill some of the claims. Therefore, further extensive research work must evaluate the beneficial effects of fungal probiotic candidates as well as identify and characterize new potential fungal strains as probiotics from different functional food sources and with strain-specific mechanisms of action. Standardization of the optimal dose against different adverse health conditions, bioavailability, percentage of viability, and finally safety assessment of probiotics must be accomplished in a standard trial experiment before commercial exploitation.

In addition, appropriate scientific protocols should be followed for better production, handling, and packaging before reaching the marketplace. Most potential fungal probiotics will be incorporated in food items such as energy drinks, juices, cereals, and certain medicinal foods as dietary adjuncts and made available I expensively in the public domain. Exploration of fungal-based probiotics such as *Saccharomyces boulardii* showed positive results in all the randomized clinical trials. However, the mixture of probiotic strains proved superior over use of a single strain. Many researchers have tried to increase the bioavailability and intestinal delivery of *S. boulardii* by microencapsulation or by immobilization in a matrix to maintain a healthy gastrointestinal balance to improve human health. Several easily biodegradable and biocompatible matrices are used for probiotic encapsulation, such as gelatin, carrageenan, chitosan, starch, pectin, or cellulose derivatives and other synthetic monomers. Encapsulation increases the bioavailability of probiotics and thus their effectiveness in the treatment and prevention of several gastrointestinal disorders. Future studies will disclose the ultimate potential of fungal probiotics in different applications.

3.7 Conclusion and Future Prospects

A fungal probiotic has been used effectively in the management of different types of gastrointestinal disorders and also in several adverse health conditions. Advanced molecular phylogenetic study clearly confirmed that *Saccharomyces boulardii* is a unique fungal strain of *Saccharomyces cerevisiae* with potential probiotic characters. Several other strains of yeast also fulfilled the required probiotic properties. The exact mechanism of action of those identified fungal probiotics has not been completely elucidated, and therefore further research is needed for this concern. In

vitro and in vivo studies will ultimately confirm the beneficial effects of fungal probiotics and also its therapeutic approach for the prevention and treatment of several diseases.

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Chapter 4
Fungal White Biotechnology Applications for Food Security: Opportunities and Challenges

Surekha Challa, Titash Dutta, and Nageswara Rao Reddy Neelapu

4.1 Introduction

Food security and food insecurity is under the purview of Food and Agriculture Organization (FAO) of the United Nations, and the concept or definition has been changing right from the World Food Conference conducted in 1974 till date. Food security means when "all people, at all times, have physical, social and economic access to sufficient, safe and nutritious food which meets their dietary needs and food preferences for an active and healthy life. Food insecurity exists when people do not have adequate physical, social or economic access to food as defined above" (Fig. 4.1) (FAO 2003). Literature reports that availability, access, utilization and stability are the four facets of food security. Availability means physical availability of food in the form of stock to trade. Access to food means adequate food supplies which are based on policies, even when market prices are not affordable by the people. Food utilization means household distribution to individuals for effective biological utilization. Stability means perpetuity of the other three facets of food security (like availability, access and utilization) even when climate, political or economic factors (like employment and market) are not in support (De Schutter 2014). The approaches implemented to ensure food security are "World Food Programme" by FAO of the United Nations, global partnerships, increase in agricultural productivity, large-scale storage of food, agricultural insurances and others (Joachim et al. 2003; De Schutter 2014; WFP 2009; Fan et al. 2015; Fan and Polman 2014; Molden 2013; McCullum et al. 2005; Delang 2006). The challenges associated with food security are water crisis globally, degradation of land, change in climate,

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Fig. 4.1 (**a**) The four facets of food security and (**b**) the three types of food insecurity

agricultural diseases, fuel, politics and food sovereignty (Fraser 2003; Brown 2004, 2006; Fraser 2007; MacKenzie 2007; McKie and Rice 2007; Sample 2007; Harvey 2011; Maher and Baum 2013; Vincent et al. 2013; De Leeuw et al. 2014; Semenza 2014). The risks or factors affecting food security are population growth, dependence on fossil fuels, homogeneity in the supply of food globally, pricing of food, change in the use of land, global catastrophes and subsidies to agricultural in various countries (Pimentel and Giampietro 1994; Alley et al. 2003; Bostrom and Cirkovic 2008; Walt 2008; Pfeiffer 2009; Sankin 2013; Khoury et al. 2014; Kong 2014; Larson 2014; Oaklander 2016).

Based on the duration, food insecurity is classified into three types – chronic, transitory and seasonal. Chronic food insecurity is "long-term or persistent", whereas transitory food insecurity is "short-term and temporary". Seasonal food insecurity falls between chronic and transitory food insecurity, often associated with climate, cropping patterns, work opportunities and disease" (FAO 2008). The United States developed the following measures to describe food security or food insecurity: (1) Household Food Insecurity Access Scale (HFIAS); (2) Household Dietary Diversity Scale (HDDS); (3) Household Hunger Scale (HHS); and (4) coping Strategies Index (CSI) (Maxwell 1996; Oldewage-Theron et al. 2006; Swindale and Bilinsky 2006a; Swindale and Bilinsky 2006b; Coates et al. 2007; Maxwell et al. 2008; Ballard et al. 2011). Literature reports the role of fungi as a dietary food as well as in food and feed processing industries. So, fungi can be a boon for food directly or indirectly playing an important role in food security. This book chapter discusses the opportunities and challenges of fungal white biotechnology applications for food security.

4.2 Fungal White Biotechnology

Employing fungal enzymes or live fungi for industrial or other applications is known as fungal white biotechnology. The applications in fungal white biotechnology include biocontrol, biomass degradation, bioremediation, bioenergy (biofuel), chemicals (organic acids), detergents, enzymes, food and feed, proteins, paper and pulp, pharmaceuticals (antibiotics, secondary metabolites, statins) and textiles (Fig. 4.2). Recently, fungal white biotechnology has gained importance due to its eco-friendly nature or ability to bring down greenhouse gas emissions. Fungal white biotechnology applications can be categorized into the following.

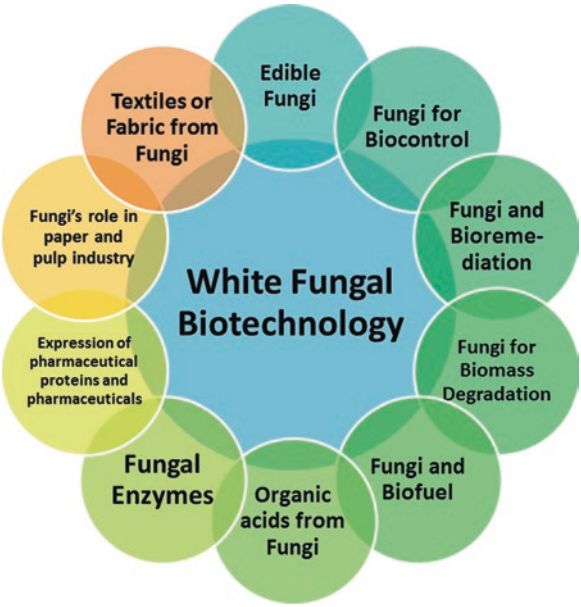

Fig. 4.2 White fungal biotechnology applications

4.2.1 Fungi's Role in Food Industry

Fungi have great and potential applications in the food industry as food (edible fungi), processed food (bread, cheese and other bakery products), fermented foods (alcohols, beverages), fodder, etc. (Ghorai et al. 2009).

4.2.2 Fungi for Biocontrol

The other potential application of fungi is biocontrol. Fungi belonging to the order *Hypocreales* are used to control pests (insects or phytopathogens) (Neelapu et al. 2009). *Beauveria bassiana*, *Nomuraea rileyi, Metarhizium anisopliae* and *Paecilomyces fumosoroseus* (mitosporic or asexual or conidiogenous entomopathogenic fungi) are the potential biopesticides available in the market to control insects (Padmavathi et al. 2003; Devi et al. 2006; Devi et al. 2007; Neelapu et al. 2009; Verma et al. 2017), whereas biocontrol agent *Trichoderma viride* is used to control phytopathogens (Surekha et al. 2013; Surekha et al. 2014; Rao et al. 2015).

4.2.3 Fungi and Bioremediation

The other promising application of fungi is bioremediation. Solid evidence was reported on different fungi possessing the ability to degrade or remediate industrial or paper mill or textile dye effluents (Lalitha et al. 2011).

4.2.4 Fungi for Biomass Degradation

Filamentous fungi produce enzymes which can degrade plant materials like hemicellulose, lignocellulose, lipids, pectin, protein and starch. Biomass degradation or conversion is another capability of fungi to convert agri-wastes or wastes into useful products for aesthetic beauty of the environment (Lalitha et al. 2013; Shukla et al. 2016).

4.2.5 Fungi and Biofuels

A fuel (bioenergy) alternate to fossil fuel is derived from plants or microalgae or from "wastes of agricultural, commercial, domestic, and/or industrial wastes". The above biomass or wastes are converted into biodiesel or bioethanol in the presence of yeast. This biodiesel or bioethanol (fuel) are additives to diesel or gasoline which can reduce emissions (Sergeeva et al. 2008).

4.2.6 Organic Acids from Fungi

Organic acids such as citric acid, fumaric acid, itaconic acid, malic acid and succinic acid have a wide range of applications in beverage, chemical, food, pharmaceutical and polymer industries. Citric acid is the most widely used organic acid in industries for the production of beverages, food and pharmaceuticals. Itaconic acid is used in the chemical industry for the production of fibres, oil additives, plastics, rubbers, surfactants and synthetic resins. Fumaric acid, malic acid and succinic acid are used in manufacturing biodegradable polymers (Meyer et al. 2016).

4.2.7 Fungal Enzymes

Fungi are the potential source of enzymes with a broad array of applications in industries. The diverse groups of enzymes from different groups of fungi have been reported with potential applications in agriculture, food and medicine industry (Kaur et al. 2017; Kumar et al. 2017; Suman et al. 2016; Yadav et al. 2017, 2018). Enzymes calf renin (chymosin) have a role in cheese production, whereas enzymes like lipases are used in the field of biomedical sciences, chemical industries, detergent industry, food technology and beverages (clarification of fruit juices). Lipases in combination with other enzymes like oxidases, peroxidases and proteases are used as household detergents and industrial cleaners and also in leather processing (Adrio and Demain 2003).

4.2.8 Expression of Pharmaceutical Proteins and Pharmaceuticals

Apart from bacteria, fungi are always considered as safe and best for the production of pharmaceutical proteins and pharmaceuticals. Few pharmaceutical proteins which are produced in fungi are fusion proteins like human interleukin-6; human lactoferrin; human lysozyme; hirudin (a thrombin inhibitor); human epidermal growth factor; human haemoglobin; human interferon; and surface antigens of the hepatitis B virus. Fungi are a good source for many known pharmaceuticals such as antibiotics, secondary metabolites, statins, immunosuppressive agents, hypocholesterolaemic agents, antitumour agents, mycotoxins, pigments and polyunsaturated fatty acids (Adrio and Demain 2003).

4.2.9 Fungi's Role in Paper and Pulp Industry

Wood is initially chipped, debarked, steamed and reduced into pulp. The pulp or raw material in the presence of fungi is converted into paper with the help of cellulose-degrading enzymes (Jerusik 2010).

4.2.10 Textiles or Fabric from Fungi

The futuristic fabric which is anti-microbial, biodegradable, comfortable to wear, durable, eco-friendly, fire-resistant, flexible, non-toxic, skin-friendly, strong, suitable for sensitive skin, waterproof and can be mended easily is now available from fungi (Ross 2016). Fabric from fungi is an innovation in textile industry and a possible substitute for animal leather and suede that can be used for accessories, bags and shoes. This fabric or leather is made by a process known as bio-fabrication (Ross 2016). MycoTEX and Muskin are made from roots and vegetable parts of mushrooms, respectively. Roots or vegetable discs of mushrooms are supplied with nutrients or tree mulch or agricultural waste or raw material in a Petri dish, allowing the root to grow in a process which is similar to fermenting. Then the dress can be mended without sewing, just by overlapping the root discs to form a thin fabric that is shaped into a dress on a mannequin. Tears and holes are simply patched by placing patches of mycelium discs over the hole (Ross 2016). The challenge with fabric made from fungus is that the manufacturing process is time-consuming and laborious, making commercial-scale production difficult. At the same time, the raw material used to grow fungi is waste, which may bring aversion to the consumer (Ross 2016).

4.3 Fungal White Biotechnology Applications for Food Security

The opportunities and challenges of white fungal biotechnology are elaborated and discussed in detail. This section discusses (1) fungi and food, (2) processed food, (3) fermented alcohols and non-alcoholic beverages, (4) fungi and pigments, (5) yeast and fodder and (6) the role of fungal enzymes in the food industry.

4.3.1 Fungi and Food

Fungi can directly be used as food (mushrooms as the edible fungi) or can be used to produce protein biomass such as SCP. This section deals with the above two aspects.

4.3.1.1 Mushrooms as Edible Fungi

Mushrooms are the integral ingredients in major cuisines worldwide due to their characteristic flavour, nutraceutical properties, and ecological and economic significance. Mushrooms also function in the prevention and treatment of many diseases. In nature 2000 species of mushrooms exist; and among these 33 are commercially cultivated for human consumption (Table 4.1) (Miles and Chang 2004; Sánchez 2004). Mushrooms are highly nutritious with considerable organoleptic and medicinal properties. Generally, they are composed of water (90%) and dry matter (10%). Mushrooms are good sources of protein (27–48%), carbohydrates (60%) and lipids (2–8%) (Valverde et al. 2015). Additionally, they are also rich in amino acids such as leucine, valine, glutamine, glutamic and aspartic acids and vitamins such as B1, B2, B12, C, D and E (Mattila et al. 2001; Surekha et al. 2011; Kalač 2013). The total energy generated per kg of fresh mushrooms is between 1.05 and 1.50 J. *Agaricus bisporus* (button mushrooms) is the most cultivated mushroom at industrial scale followed by *Lentinus edodes* (shiitake), *Pleurotus* spp. (oyster mushrooms), *Auricula auricula* (wood ear mushrooms), *Flammulina velutipes* (winter mushrooms) and *Volvariella volvacea* (straw mushrooms) (Aida et al. 2009; Cheung 2013). Apart from their nutritional values, many species of mushrooms are traditionally used as medicine due to their immunomodulatory and antineoplastic properties. They are widely accepted for their antiallergic, antibacterial, anticancer, anticholesterolaemic, antidiabetic, antifungal, anti-inflammatory, antiparasitic, antioxidant, antitumour and antiviral properties. They are also known for their cardiovascular, detoxification and hepatoprotective effects (Wasser 2014).

Tian et al. (2018) identified white button mushroom (*A. bisporus*)-mediated effect on glucose homoeostasis via intestinal gluconeogenesis. Thus, white button mushroom has a prebiotic role on glucose homeostasis, probably controlling diabetes.

Table 4.1 List of edible mushrooms available for human consumption

S No.	Scientific name	Common name
1	*Naematoloma sublateritium*	Brick cap
2	*Hericium erinaceus*	Lion's mane or bear's tooth
3	*Entoloma abortivum*	Aborted entoloma
4	*Morganella pyriformis*	Pear-shaped puffball
5	*Lepista nuda*	Blewits
6	*Flammulina velutipes*	Velvet foot
7	*Coprinus comatus*	Shaggy manes
8	*Agaricus campestris*	Meadows or pink bottoms
9	*Armillaria gallica*	Brown honey or stumpers
10	*Armillaria mellea*	Yellow honey or stumpers
11	*Grifola frondosa*	Hen of the woods
12	*Pleurotus ostreatus*	Oyster mushrooms
13	*Laetiporus cincinnatus*	Chicken of the woods
14	*Lepiota americana*	Reddening *Lepiota*
15	*Pluteus cervinus*	Deer or fawn mushrooms
16	*Suillus americanus*	Chicken fat
17	*Auricularia auricula-judae*	Woodears
18	*Phylloporus rhodoxanthus*	Gilled bolete
19	*Boletus edulis*	King bolete
20	*Russula virescens*	Green quilted *Russula*
21	*Lentimula edodes*	Shiitake
22	*Panellus serotinus*	Late fall oysters
23	*Hypomyces lactifluorum*	Lobster mushrooms
24	*Pleurotus porrigens*	Angel wings
25	*Laccaria ochropurpurea*	Purple-gilled *Laccaria*
26	*Macrolepiota rhacodes*	Shaggy parasol
27	*Volvariella volvacea*	Straw mushrooms
28	*Tremella fuciformis*	Silver ear
29	*Agaricus bisporus*	Button mushrooms, champignon
30	*Morchella elata*	The black morel
31	*Morchella esculenta*	The common morel
32	*Tuber melanosporum*	Truffle
33	*Ganoderma lucidum*	Reishi

Source: Miles and Chang (2004)

C57BL/6 mice when fed with white button mushrooms showed significant changes in the composition of microbiota (especially significant increase in *Prevotella* population). *Prevotella* produce propionate and succinate which induce intestinal gluconeogenesis probably influencing the gut-brain neural circuit, thereby decreasing hepatic glucose in C57BL/6 mice. The edible mushroom *Lentinus edodes* (Shiitake) produces lentinan, a fungal polysaccharide with immense medical properties. The primary chain of lentinan is composed of β-(1,3)-D-glucose units linked to β-(1,6)-D glucose side groups with an average molecular weight of 500,000 Da (Morais et al.

2000). Similarly, the polysaccharide schizophyllan (sizofiran) obtained from the edible mushroom *Schizophyllum* has a molecular weight between 100,000 and 200,000 Da and exists in a triple helical conformation. These polysaccharides are extensively used as immunomodulators and therapeutics for cancer. Another edible mushroom *Agaricus blazei* (grown in Brazil) is reported with significant antitumour properties. *Cordyceps militaris* is another mushroom grown at a large scale due to its multiple medicinal benefits. This mushroom is known for antibacterial, antimetastatic, antiproliferative, antitumour and anti-inflammatory properties, along with potent immunomodulatory and insecticidal effects. *C. militaris* when consumed supresses chronic bronchitis, influenza A and viral infections (Won and Park 2005).

The cultivation of mushroom begins with the procurement of pure mycelium from the spores of the desired or specific mushroom strain or from a piece of specific mushroom. The next stage is obtaining inoculum from the pure mycelium by growing it on spawn (cereal grain, e.g., wheat, rye or millet) (Chang and Miles 1989; Oei 2003). The production of mushroom depends on the quality of spawn, and therefore spawn is prepared in sterile conditions to prevent contamination. Several groups have developed new techniques to enhance or produce high-quality spawns (Flegg and Maw 1976; Amuneke and Dike 2017). The species of mushrooms to be cultivated determines the degree of substrate preparation, inoculation, incubation and production conditions. For example, *L. edodes* (shiitake mushrooms) which is traditionally grown on wooden logs is now grown on artificial log cultivation or bag-log cultivation (utilizing heat-treated substrates enclosed in plastic bags) (Chang and Miles 1989). This new technique is more advantageous than the traditional method of cultivation, as the time interval for a complete crop cycle is significantly reduced, along with scaling up mushroom production. *Pleurotus* spp. (oyster mushrooms) are not grown on wooden logs; instead, they are grown on a wide range of lignocellulosic materials. The materials used for growth are pasteurized before inoculation to reduce contamination and enhance the quality of the mushroom (oyster mushroom).

Most of the mushroom species are good sources of bioactive compounds apart from their nutritional value. The growing awareness related to the nutritional and nutraceutical properties of mushrooms has drastically increased the consumption of whole mushrooms as dietary supplements. This potential demands an increased and sustained production of edible mushrooms to meet the growing demand worldwide. Modern technologies such as computerized control systems to monitor environmental parameters, automated harvesting and mushroom production using non-composted substrate, novel substrate sterilization, spawn preparation techniques, etc. are used for industrial-scale production. Mushroom production using non-composted substrate is highly recommended as the cropping cycle of the mushroom can be reduced along with production cost. Moreover, the odour generated during the composting process, which is a potential environmental hazard, is also reduced. Similarly, developing novel strains will enhance the yield, disease resistance and increase productivity.

4.3.1.2 Single-Cell Protein

The world population grows continuously posing a significant challenge towards providing an adequate food supply for all of the growing population. Scientists are in the search of novel and alternative protein resources to meet the demands of the growing population. Bacteria, algae, fungi and yeast are used to produce protein biomasses. Carol L. Wilson in 1966 named these protein biomasses as single-cell protein (SCP) (Suman et al. 2015). SCP can be defined as the mass of dried microorganism cells with enriched protein content (about 30–50%), fats, carbohydrates, nucleic acids, vitamins (B-complex vitamins: thiamine, riboflavin, biotin, niacin, pyridoxine, etc.), minerals (Ravindra 2000; Adedayo et al. 2011; Nasseri et al. 2011) and fibres used for both human consumption and as animal feed. SCPs also contain certain essential amino acids like lysine and methionine at higher levels, which are limited in most plant and animal foods. The supplementation of protein in a diet is important as essential amino acids are irreplaceable. Various groups have explored for protein supplements with essential amino acids (like methionine and tryptophan) as plants lack these essential amino acids and the conversion of plant biomass into proteins by animals is low. Microorganisms like bacteria, yeast, fungi and algae act on inexpensive feedstock and waste products for the production of SCP. These microorganisms possess high growth rate and can be genetically engineered to develop novel strains capable of enhancing protein quality as well its protein content.

The substrate used for conventional production of SCPs includes starch, molasses, fruit and vegetable wastes. Recently, SCPs are also produced from non-conventional substrates such as petroleum by-products, natural gas, ethanol, methanol and lignocellulosic biomass (Lenihan et al. 2010). The major filamentous fungal species used for commercial production of SCPs include *Chaetomium cellulolyticum, Fusarium graminearum* (Zubi 2005), *Aspergillus fumigates, A. niger, A. oryzae, Cephalosporium cichorniae, Penicillium cyclopium, Rhizopus chinensis, Scytalidium acidophilum, Trichoderma viride* and *T. alba* (Bajpai 2017).

SCPs are commercially available as products for human consumption. SCP produced from the fungi *F. venenatum* reduces LDL cholesterol and blood glucose and regulates insulin (Ugalde and Castrillo 2002; Gabriel et al. 2014). Development of the product Quorn mycoprotein from *F. graminearum* is a breakthrough, targeting human nutrition, and was exclusively marketed and sold across the United Kingdom for human consumption. Quorn is popular due to its meat-like texture and appearance (Garodia et al. 2017). Similarly, brewer's yeast extract from *Saccharomyces cerevisiae* is a good protein source and is used to develop a variety of products such as Marmite, Vegemite, Cenovis and Vitam-R. Another product Torula, from *Candida utilis* (a popular yeast extract), has high glutamic acid content and is used commercially as a flavouring agent in place of monosodium glutamate (MSG) (Olvera-Novoa et al. 2002).

SCPs for animal livestock are produced by the action of the filamentous fungi *Paecilomyces varioti* and *Yarrowia lipolytica* strains. In Finland, *P. varioti* strains

were grown on sugar-rich medium derived from wood hydrolysates or sulphite containing effluents from paper mills, and this process is known as "Pekilo" (Bajpai and Bajpai 1987). The other commercially viable substrate where *Y. lipolytica* strains were grown to produce SCPs is n-paraffin wax (Papanikolaou et al. 2007). Though a majority of the fungal species are not detrimental for humans, there are some species such as *A. niger*, *A. fumigates* and *F. graminearum* which are toxic. Therefore, such fungi should be avoided or subjected to toxicological evaluations before recommending them as SCPs (Ukaegbu-Obi 2016).

Moreover, significant research is carried out to produce SCPs with enhanced content and quality of protein using different modes of fermentation (such as solid-state fermentation, submerged fermentation, etc.). To avoid spoilage, SCPs are dried by eliminating excess moisture and are then concentrated by using filtration, coagulation and precipitation techniques. These steps led to stability and enhanced shelf life of the SCPs produced, thereby increasing their storage capacity.

4.3.2 Processed Foods

The role of fungi in food processing industry is well known. In this section the details of fungi used in the processing of cheese and bread are discussed.

4.3.2.1 Cheese and Role of Fungi in Cheese Processing

History dates the practice of producing cheese to ancient times; and the cheese industry has bloomed into a multimillion dollar venture across the United States and other countries are catching up this trend. The discovery of cheese was accidental, where milk carried in a pouch made of sheep's stomach curdled over a period. Cheese is an excellent source of vitamins and calcium in our diet. Additionally, cheese adds a wide variety of flavours and texture to a palate. The general procedure for the production of cheese involves fermenting milk of animals in the presence of various fungal and bacterial species. The milk for fermentation is collected from various animals like cow, goat, ewe, sheep, etc. Fermented milk is coagulated using the enzyme renin, followed by the removal of the liquid whey. Subsequently, the solid curd is processed and preserved under controlled conditions (environment and temperature). Commercially, different varieties of cheeses are available for human consumption. These cheeses are soft cheeses, blue-veined cheeses, hard cheeses, uncooked firm cheeses and cooked firm cheeses.

The variation of cheese depends on texture and flavour, which is contributed while processing cheeses. The differences in texture and flavour of cheeses depend on various mechanical techniques like carving, brewing, pressing, grinding and heating which are utilized to drain the curd (McSweeney and Sousa 2000). The development of flavour, texture and nutritional aspects of cheese are linked to the degradation of proteins and lipids of milk (Sousa et al. 2001). The development of

unripened cheese requires adding minute amounts of salt to coagulated milk; and later the settled curd is cut, drained and washed. Salting improves and is responsible for the development of different cheese varieties with improved quality, texture and flavour. Unripened cheese (e.g., cottage cheese) has a shorter shelf life due to high moisture content (60–80%) and is preserved under chilled temperatures to maintain the required moisture content. To increase the storage capacity, scientific groups have focussed on developing dry cheeses (parmesan) and hard cheeses (cheddar) by lowering the moisture content significantly to 32% and 35%, respectively.

Filamentous fungi play a central role in cheese making and contribute towards the organoleptic properties of cheese. The species used for cheese ripening and developing different varieties are *P. camemberti*, *P. roqueforti*, *Mucor fuscus*, *M. lanceolatus*, *G. candidum*, *F. domesticum*, *Sporendone macasei*, *Scopulariopsis flava* and *S. fusca* (Hermet et al. 2012; Ropars et al. 2012). These fungal strains are either present in raw material (milk) or inoculated during the cheese-making process. These strains enhance appearance, texture and flavour of cheeses. The two most commercially popular varieties of cheeses are Camembert and Roquefort (blue cheese). The other commercially available are blue cheeses (Danish blue), Stilton cheese, Gorgonzola cheese and Limburger cheese. All cheeses require fungal fermentation, whereas limburger cheese is fermented in the presence of both fungi and bacteria and exhibits a strong flavour and aroma. Camembert cheese is marketed as Brie with moderate moisture content (40–50%) and is produced by eliminating whey and placing curd solids in disc-shaped containers. Moulds like *P. camemberti* and *P. casicolum* enhance the appearance, flavour and texture of Camembert cheese through lipolytic and proteolytic activity (Yadav and Mishra 1995). The production of Camembert cheese also requires rigidly controlled conditions to prevent the growth of other fungi (particularly *S. brevicaulis*) and subsequent spoilage of cheese. Roquefort cheese is produced using the fungal strain *P. roqueforti* to enhance the appearance, texture and flavour of blue cheese (Beresford et al. 2001; Metin 2018).

4.3.2.2 Bread and Role of Yeast in Bread Making and Processing

Bread is a baked product prepared from dough and yeast along with a specific flavour and texture (Querol and Fleet 2006). Bread is an integral component of food in almost all successive human civilizations. Archaeological data (dating 10,000 years ago), collected from the sediments of a Swiss lake dweller, shows the association of humans with bread making. The fungal culture (yeast) or other gas-forming organisms are added to the bread dough and mixed thoroughly to initiate rapid sugar fermentation. The chemical reaction due to sugar fermentation in the dough produces acid and alcohol, facilitating the production of carbon dioxide. This gas (CO_2) raises the bread slowly, giving the characteristic raised appearance (leavened) to bread and also with airy texture of bread. At the same time, some metabolites escape from the bread, while some metabolites are retained in the bread developing flavour. The microorganism used in bread is popularly known as baker's yeast (a strain of *S.*

cerevisiae). This yeast is designed specifically to enhance gas formation, viability during storage, and the ability to generate a desirable flavour in baked products. In late nineteenth century, baker's yeast was exclusively developed for baking industry. Baker's yeast was grown on mashed grains (sources of sugar), whereas molasses are used as an inexpensive source for assimilable sugars. The gradual evolution of techniques used in baking industry put forward a number of challenges to yeast strains. The challenges faced by yeast strains while baking are (1) tolerance to high sugar concentration (in sweet dough); (2) tolerance to drying and freezing (in relation to the increase of the production of dry and frozen dough); and (3) improving sugar fermentation efficiency.

4.3.3 Fermented Alcohols and Beverages

The present food world is preoccupied with fermented alcohols and beverages (wine, beer, sake, chichi, shoyu, tempeh, injera, etc.) and non-alcoholic beverages like apple cider. In this section the role of fungi in the preparation and processing of fermented alcoholic and non-alcoholic beverages is discussed.

4.3.3.1 Beer and Clarification of Beer

4.3.3.1.1 Production of Beer

Beer is one of the most popular alcoholic beverages worldwide; and its total consumption is 276.4 billion litres in 2017 (Bamforth 2017). Beer is produced using a mixture of water, hops, malt and yeast, whereas stout beers are produced from roasted barley or malt (Saerens et al. 2017). Hops is responsible for the characteristic bitterness and aroma of beers. Malt obtained from barley grains provides enzymes, and these enzymes are required for the degradation of starch and proteins into simpler forms that can easily be used by the yeast. The fermentation process is stopped by eliminating the water in malts either by applying fresh air or heat or changing them into dried malts. When fresh air is used, the colour of the dried malts is green, whereas in the presence of heat, the colour intensifies to produce dark green malts leading to the dark colour of beer. In general, the yeasts belonging to the genus *Saccharomyces* are employed in beer production. However, other strains such as *S. pastorianus* and *S. eubayanus* are also currently employed for the production of beer. According to the physical characteristics, there are two main varieties of beers available – ale or lager-style beers. Ales are prepared by fermenting malt with strains of *S. cerevisiae* at a high temperature range (15–26 °C) near the surface of the fermenting wort. In the case of lager beers, the fermentation of malt with yeast strains is carried out at a relatively low temperature range (5–14 °C) near the bottom of the fermenter. During the stages of mashing and brewing, enzymes are added to

catalyse the brewing process. Enzymes such as amylase, improve the digestion of starch resulting in low carbohydrate or "light" beers. Other enzymes like protease and glucoamylases are added to make beers hazy and sweet, respectively (Souza 2010; Brányik et al. 2012).

4.3.3.1.2 Clarification of Beer

Laccases are used for stabilizing beer, a popular alcoholic beverage. Haze formation in beer is due to protein precipitation initiated by proanthocyanidins and polyphenols present in beer (Mathiasen 1995). This phenomenon is referred to as chill haze, which takes place when the beer is cooled. The haze formation usually re-dissolves as the beer reaches room temperature or above. But over a long time, the protein sulphhydryl groups substitute the phenolic rings leading to permanent haze formation (Minussi et al. 2002). Traditionally, polyphenols are eliminated by polyvinylpolypyrrolidone (PVPP) treatment, but PVPP is toxic and interferes with wastewater treatment due to its low biodegradability. To overcome this problem, treatment with laccase is recommended as it is nontoxic, easier to handle and efficiently removes polyphenols in worts (Minussi et al. 2002). The addition of laccase during processing successfully eliminates polyphenols, and at the same time low oxygen content enhances the shelf life of beer (Mathiasen 1995).

4.3.3.2 Wine and Clarification of Wine

4.3.3.2.1 Production of Wine

Wine, a popular alcoholic beverage, is basically a fermented juice of grapes. The Biblical account of Noah (5000 BC) provides details on the making of wine, dating wine to the earliest history of man. Wines are named after the varieties of grapes used for production or after their location or the area where it was first produced. For example, Burgundy, Bordeaux, Champagne and Alsace are important wines of France. There are three basic types of wines: (1) table wine, (2) fortified wine and (3) sparkling wine (Amerine 1980). Table wine (12–15% alcohol content) is produced by pressing grapes and then allowing the mixture to ferment in vats along with sugar, yeasts and sulphur dioxide. *S. ellipsoideus* is the common yeast strain used in the fermentation process. Port wine (19–20% alcohol content) is a common fortified wine, where its name is derived from the sailors who frequented the ports to purchase wines. These wines were spiked with other alcohols like brandy to increase the alcohol levels. Sparkling wine (champagne) is prepared by double fermentation, where the alcohol content is increased to 20%. Some sparkling wines have a natural effervescence due to fermentation, while others are made effervescent by adding carbon dioxide (Torresi et al. 2011). All natural wines have alcohol content less than 20%, to ensure proper functioning of yeast during fermentation.

Wines are also classified into red, white and pink wines based on their appearance or colour. Red wine is produced from black grapes along with their skins, whereas white wine is made from green grapes or black grapes devoid of skin (after pressing). Pink wines are produced when black grapes with skin are incubated in the fermenter for a short period (Jackson 2008).

The fermentation of wine is a complex process involving many yeast genera and species (Guillamón et al. 1998). In the first stage of fermenting wine, non-*Saccharomyces* yeasts are added, and later, *S. cerevisiae* is used for degrading sugars due to its high fermentation efficiency and tolerance to ethanol. As the fermentation process progresses, yeast strains come in contact with major stress conditions. The stress conditions are high osmolarity, high sugar concentrations (180–260 g/l), low pH (3–3.5), low sulphites (40–80 mg/l), low oxygen and nutrient content (nitrogen, lipids and vitamins) and toxicity to the end product (ethanol). During fermentation, a majority of sugars present in the form of hexoses are converted into ethanol and CO_2, while the remaining sugars in small amounts carry the synthesis of anabolic precursors necessary for producing biomass. The major phases in wine fermentation are lag phase, short growth phase followed by a stationary phase. During the stationary phase, a majority of the sugar molecules are fermented. The limiting nutrient nitrogen is responsible for the arrest of cell proliferation, while other limiting micronutrients include lipids and vitamins.

Presently, there are more than 200 strains of *S. cerevisiae* used in wine industries to produce wine. These strains are selected based on spontaneity in fermentation or performance associated with specific vineyard environments (Guillamón et al. 1998). In addition to *S. cerevisiae*, other *Saccharomyces* species and a number of interspecies hybrids involved in wine fermentation are recently identified and characterized. Interspecific hybrids include *S. cerevisiae/S. kudriavzevii* (González et al. 2006, 2008), *S. cerevisiae/S. uvarum* (Naumov et al. 2000; Sipiczki 2008) and *S. cerevisiae/S. kudriavzevii/S. uvarum* (Lopandic et al. 2007; Masneuf et al., 1998). These hybrids are more efficient than their wild types and are associated with increased tolerance to various stresses (Le Jeune et al. 2007).

Although wine industry is immensely benefited with strains of superior and desirable traits, major research is still in progress to improve wine yeasts for various traits. These traits include stress tolerance, fermentative performance, aroma and ethanol tolerance. Developments in the field of molecular biology, assisted with the latest analytical techniques such as quantitative trait locus (QTL), aided in the identification of desirable traits from the genome of yeast. QTLs associated with the formation of acetic acid, aroma enhancement, SO_2 production, nitrogen utilization and ethanol tolerance are identified (Roncoroni 2014). These QTLs can be used to engineer the desired alleles and transform them into novel strains using techniques like marker-assisted allele transfer. These strategies have the potential to develop superior yeast strains with enhanced ethanol resistance, which are capable of overproducing esters that contribute the fruity aroma to wines (Van Rensburg et al. 2005).

4.3.3.2.2 Clarification of Wine

During the crushing and pressing stages of wine production, a high concentration of phenolics and polyphenols was observed. The stems, seeds and skins have a high concentration of tannins and polyphenols that contribute to its colour and astringent taste, depending on the variety of grape and the vinification conditions (Minussi et al. 2007). Natural oxidation of these polyphenols and tannins alters the flavour and colour in red wines. Minussi et al. (2007) observed that treatment with laccase significantly removed polyphenols and enhanced the organoleptic characteristics of wines while protecting its distinct taste and colour. Moreover, laccases also improved the shelf life of wines when stored for longer time periods.

4.3.3.3 Other Alcoholic Beverages

Sake (rice wine) is a traditional alcoholic beverage prepared from rice and is particularly consumed in Japan and China (Blandino et al. 2003). The rice is polished, steamed and is fermented in the presence of *A. oryzae*. *A. oryzae* produces different types of enzymes required for sake brewing. The fungus converts the starch into simpler sugars, where these simpler sugars can be used by the yeast to produce sake. The seed mash is traditionally obtained by natural lactic acid fermentation involving various aerobic bacteria, wild yeasts, lactic acid bacteria and sake yeasts (Caplice and Fitzgerald 1999; Kuribayashi et al. 2017; Terasaki et al. 2018).

Chicha is a another fermented alcoholic beverage produced from corn and is widely consumed in South America (Steinkraus 1983; Hayashida 2008). A unique fermentation process is used for the preparation of chicha, where saliva serves as the source of amylase for the conversion of starch to fermentable sugars (Puerari et al. 2015). Yeasts, particularly *S. cerevisiae,* and bacteria of the genus *Lactobacillus* sp., *Leuconostoc* sp., *Acetobacter* sp. along with various moulds such as *Aspergillus* sp. are the primary fermenting microorganisms used in the preparation of chicha (Tamang et al. 2016).

4.3.4 Other Fermented Food Products

The consumption of fermented food and beverages has been in practice since ages. Though certain fermented products like beer, wine, etc. have gained popularity, other traditionally fermented food items are not in limelight. Some examples of these food products are miso, shoyu, tofu, injera and tempeh (Soni and Dey 2014). Significant research is also carried out worldwide to identify the microorganisms involved in the fermentation of these products and their mode of action.

Shoyu or soy sauce is a dark brown liquid made from a blend of soybeans and wheat, which is used as seasoning or flavouring agent in Japan, China and the Far

East countries (Fukushima 2004). Shoyu has a salty taste but is lower in sodium content than the traditional table salt. Soybeans are initially cooked and cooled to room temperature. Then, coarse wheat flour is added to the cooked soybeans and mixed thoroughly. The moisture content of the soybean–wheat mixture is maintained at 55% (w/w). The mixture is then inoculated with mould *A. oryzae* and fermented at 25–35 °C. After 3 days of incubation, the soybeans and flour mixture (referred to as koji at this stage) is placed in a brine solution (22–25%) and mixed thoroughly. This brine solution containing koji is known as moromi. The moromi is then inoculated with *Pediococcus soyae* (bacterium) and *S. rouxii* and *Torulopsis* sp. (yeasts) to ferment the mixture for a period of 1–12 months. The quality of soy sauce is dependent on the time taken to ferment moromi (Luh 1995). After fermentation, the liquid part (soya sauce) is separated, filtered, pasteurized and bottled. The characteristic aroma and flavour of soy sauce is imparted due to the enzymatic changes of yeasts.

Tempeh, a native dish of Indonesia (Babu et al. 2009), is produced by fermenting boiled legume seeds of soybean, peanut and mung bean with *Rhizopus oligosporus* strains and is now being explored in the United States (Buckle 1988). The seed coat of the legume is removed before the fungal culture is added; this allows the fungus to have better access to nutrient-rich cotyledons. The inoculation of the fungus aids in the breakdown of complex carbohydrates and other organic compounds that are involved in gas formation.

Miso, a fermented soybean paste, originated in Japan ~2000 years ago. It is used as a base for soup, as well as seasoning agent (Robinson 2000). The fermentation procedure consists of washing polished rice and steaming. Then rice is inoculated with *A. oryzae*, resulting in formation of "rice koji". This fungus converts the carbohydrates and proteins in rice into amino acids and sugars. The rice koji is then inoculated with yeasts and bacteria and then subjected to fermentation at 28 °C for 1 week, followed by fermentation at 35 °C for 7–12 months. All the above processes are carried out in the presence of fungal enzymes like cellulase, catalase, lipase, glucose oxidase, etc. which are then released into the respective substrates catalysing the fermentation process.

Injera, the national food of Ethiopians, is produced from different cereal sources such as sorghum, tef, corn, finger millet and barley. The grains are dehusked, converted into flour and mixed with water to form dough. This dough is allowed to ferment for a period of 2 or 3 days in the presence of a starter (ersho). The starter is a fluid-like substance preserved from the previous fermented dough. After fermentation, a thick batter of dough is prepared and poured in an oil grease pan fitted with a tight lid. This pan is allowed to steam for 2–3 min and stored in a basket. The storage period of injera is not more than 3 days at room temperature. The major microorganisms associated with injera fermentation are yeasts and fungi (*Pullaria* sp., *Aspergillus* sp., *Penicillium* sp., *Rhodotorula* sp., *Hormodendrum* sp. and *Candida* sp.) (Girma et al. 1989; Stallknecht et al. 1993; Ma 2012). A typical injera is round in shape, has a soft and spongy texture and distinct light sour flavour and measures about 6 mm in thickness and 60 cm in diameter (Gebrekidan and Gebrehiwot 1982). Injera has a very high nutritional value, particularly enriched with calcium and iron (Mohammed et al. 2011).

4.3.5 Non-alcoholic Beverages

In the above section the role and details of fungi involved in producing fermented alcoholic beverages were discussed. In this section the role and details of fungi involved in the production of non-alcoholic beverages like apple cider are discussed.

4.3.5.1 Apple Cider

Apple cider is native to the United States and Canada and is seasonally produced in autumn. This is a non-alcoholic beverage which is traditionally served on Christmas, Halloween, New Year's Eve, Thanksgiving and various other holidays. Apple or apple core or apple trimmings or apple culls are used as source to press at farmsteads or local mills for extracting liquid; and this extract is boiled and concentrated. Pomace, the leftover after pressing, is used as a feed for cattle. Apple cider is pasteurized to extend shelf life of cider by killing the bacteria. Cider may also be fermented to produce hard cider and later may be treated with *Acetobacter* to produce vinegar; or apple brandy is produced by distilling.

4.3.5.2 Clarification of Cider

Apple juices are clarified and processed to remove pectin and starch before consumption. Pectinases (pectic enzymes) are added during the preparation of apple juice. They help in removing pectins and tannins from apple juice and impart the characteristic aroma to the cider. Pectinases in combination with cellulases and amylases are used to filter apple juice; enhance and eliminate the haze formation; and produce clear and amber-coloured apple juice. Haze formation results due to the polymerization of polyphenols and oxidation of proanthocyanidins. Cider pectinases isolated from fungal species *A. aculeatus A. niger*, *T. viride, P. notatum* and *Botrytis cinerea* are used for clarification of the final product.

Phenols and polyphenols are naturally present in a majority of fruit juices, and they contribute towards the taste and colour of these juices. However, these phenols and polyphenols undergo polymerization and oxidation in nature which significantly deteriorate the colour and aroma of fruit juices. Laccase is another fungal enzyme used for the clarification of apple juices. Laccases stabilize the juice, prevent the loss of nutrients and increase the shelf life of apple juice. Giovanelli and Ravasini (1993) added laccase to apple juice during the filtration process to improve its stability and appearance. The laccase treatment method was efficient in extruding the phenols present in apple juice when compared to other methods (treatment with activated coals, ascorbic acid and sulphite). Phenolic content of juices was greatly reduced with laccase treatment which restored the natural taste and colour of apple juices (Ribeiro et al. 2010).

4.3.6 Fungi as Source of Food-Grade Pigments

Food-grade dyes and pigments can be obtained from selective fungal species and have been in practice since ancient times. Filamentous fungi are considered as excellent sources of food-grade pigments. The commercially available pigments include *Monascus* pigments, Arpink red, riboflavin, lycopene and β-carotene. Red rice traditionally known as koji or ang-kak is produced when rice is fermented with *Monascus purpureus* strains (Carvalho et al. 2003). The orange pigments monascorubrin and rubropunctatin react with amino acids present in the fermentation media, producing water-soluble red pigments monascorubramine and rubropunctamine, which are responsible for the colouring of rice. The major commercially available pigments include *Monascus* pigments and Arpink red obtained from *P. oxalicum*. These red pigments are used for preparing wine, soya bean cheese, meat, etc., along with red rice, and is authorized for food used in China and Japan.

Phaffia rhodozyma is the yeast that produces maximum astaxanthins, a carotenoid in the microbial world (Frengova and Beshkova 2009). The pink colour of salmonid flesh and the reddish tinge on crustaceous shells are developed using astaxanthin pigment. Supplementation of salmonids with a diet containing this yeast induces pigmentation in the white muscle (Johnson et al. 1977). For years, *Blakeslea trispora* is used for the production of lycopene and β-carotene in Russia. In this fermentation, a fungal culture is used with a preferred ratio of minus and plus strains of *B. trispora* (of mating) to yield 17 g/L of β-carotene (Jerusik 2010; Dufosse et al. 2014). The accumulation of β-carotene is strongly linked to sexual interaction between the two mating types (strains). Trisporic acid, a hormone-like substance, is produced during mating, the major component stimulating the production of the pigment. β-carotene can also be produced from the fungi *Mucor circinelloides* and *Phycomyces blakesleeanus.*

Riboflavin (vitamin B_2) is another popular food-colouring agent which gives a characteristic yellow colour. It is used in dressings, sherbet, beverages, instant desserts and ice creams. *Eremothecium ashbyii* and *Ashbya gossypii* are known to produce riboflavin (1 g/L) by fermentation. *Aspergillus* spp., namely, *A. glaucus*, *A. cristatus* and *A. repens,* have been used to produce hydroxyl anthraquinoid compounds like emodin (yellow), physcion (yellow), questin (yellow to orange brown) and erythroglaucin, catenarin and rubrocristin (red) (Caro et al. 2015).

4.3.7 Yeast as Fodder

The term “food yeast” or “fodder yeast” was coined by Professor Jacquot and Dr. Biloraud in 1957 and is used as a food supplement for domestic livestock. They are high in protein content (41%) followed by carbohydrates (32–36%), ash (4–8%), fibre (1–8%) and fat (1%). Many enzymes are used to enhance the nutritional content of animal and poultry fodder. Recent studies show that exogenous enzymes such asphytase, amylase, β-glucanase and xylanase are added to cereal-based

fodder to enhance the utilization rate of phosphorous, starch, β-glucans and arabinoxylans, respectively, from the diet (Shishkova et al. 1979; Bedford et al. 1997; Ugwuanyi 2016). Research has also highlighted the addition of certain fibre-degrading enzymes (xylanases and cellulases) to ensure the proper utilization of dietary supplements and enhance the performance levels of animals. Therefore, treating feed with enzymes improves digestibility or palatability either by promoting direct hydrolysis or by modifying the digestion sites.

4.3.8 Role of Fungal Enzymes in Food Processing Industry

Till now this chapter has discussed the role of different fungi in the food processing industry. Now, the role of fungal enzymes in all the above processes is discussed.

4.3.8.1 Role of Fungal Enzyme Laccase in Food Processing Industry

Laccase (benzenediol: oxygen oxidoreductase) represents polyphenol oxidase with a catalytic centre composed of copper atoms, giving them their characteristic blue colour. Laccase has a plethora of applications such as bioremediation; stabilization of beverages (fruit juice, wine and beer); enhancement of general food quality; and uses in baking industry. Several fungal species such as *Gaeumannomyces graminis* (Edens et al. 1999), *Magnaporthe grisea* (Iyer and Chattoo 2003), *Ophiostoma novo-ulmi* (Binz and Canevascini 1997), *Mauginella* (Palonen et al. 2003), *Melanocarpus albomyces* (Kiiskinen et al. 2002), *Monocillium indicum* (Thakker et al. 1992), *Neurospora crassa* (Froehner and Eriksson 1974) and *Podospora anserina* (Esser and Minuth 1970) of ascomycetes produce laccase and exhibit significant laccase activity.

Texture, volume, flavour and freshness of dough are the important characteristics for bread. One of the most important enzymes used for the improvement of the above mentioned characteristics is laccase. The addition of laccase to dough initiates oxidation, resulting in a stable and strong gluten structure in dough. Laccase in dough also increases bread volume, crumb structure and softness of the final products. The resulting dough exhibits increased strength, stability and less stickiness (Minussi et al. 2002). Awareness on celiac disease (CD) compelled researchers to develop gluten-free bakery products. CD is medically an immune-mediated enteropathy due to gluten ingestion. This gluten is present in major cereal flours such as wheat, rye and barley. The focus has now shifted to oat flour and starches such as rice, potato and corn for developing gluten-free baked products (Gallagher 2009). The above starches and flours are devoid of the gluten protein matrix which is the prerequisite for dough formation and lacks the physical characteristics of wheat-based baked products. Recent products based on gluten-free oat flour along with laccase produced baked products acceptable for CD patients. When laccase and proteolytic enzymes were added to oat flour, the texture and quality of oat bread improved significantly. Chemical analysis showed that the improvement of quality

is due to β-glucan depolymerization and protein polymerization. Thus, depolymerization and polymerization result in greater specific volume of the loaf; reduced crumb hardness; and chewiness of the oat bread.

Thus, laccases have a wide range of applications in food processing ranging from developing gluten-free bakery products to restoring taste and stability in alcoholic and non-alcoholic beverages. Moreover, they significantly reduce the expense incurred during the processing of the products and also generate environment-friendly products. Although the use of laccase has increased in bakery and wine industries, a thorough knowledge of laccase production and their mode of action, as well as the efficient production of laccase units, is required to harness the potential of laccase in the food industry.

4.3.8.2 Role of Fungal Enzyme Lipase in Food Processing Industry

Lipases chemically represent triacylglycerol acylhydrolases, and their molecular weight ranges between 19 and 60 kDa. They are widespread in nature and are abundant in animals, plants, fungi and bacteria. Lipases catalyse hydrolysis of triacylglycerols to generate free fatty acids, diacylglycerols, monoacylglycerols and glycerol. Lipases have immense physiological and industrial significance. They are actively used in oleochemical, detergent, organic, leather, cosmetics and perfume industries. Moreover, they are also used in environmental management as biosensors apart from their multiple roles in the food processing industry (Pandey et al. 1999; Aravindan et al. 2007; Sharma et al. 2011). A majority of the lipases are isolated from fungi and bacteria. Fungi are more suitable sources for lipases due to their extracellular location which facilitates their extraction. The major sources of viable lipases are *A. niger, A. terreus, A. carneus, C. cylindracea, H. lanuginose* (*T. lanuginosus*)*, M. miehei, R. arrhizus, R. delemar, R. japonicus, R. niveus* and *R. oryzae* (Sharma et al. 2001). Lipase production in all these fungal species was enhanced when they were grown on media supplemented with glucose. In 1994, Nova Nordisk developed the first commercial lipase, lipolase, which was isolated from the fungus *Thermomyces lanuginosus,* and expressed it in *A. oryzae* (Prathumpai et al. 2004).

Lipases are associated with the development of flavour in dairy products and processing of foods, such as meat, vegetables, fruits, baked food items and beer. Phospholipases are used to treat egg yolk for mayonnaise and spread production; lecithin modification; and refining of vegetable oils. Phospholipase causes hydrolysis of egg lecithin, which enhances the emulsifying capacity and heat stability of egg yolk. The resulting egg yolk is deemed useful for processing custard, mayonnaise, baby foods and dough preparation. Lipases have been successfully used as catalysts for ester synthesis. The esters fabricated from short-chain fatty acids catalysed by lipase immobilized on silica beads serve as flavouring agents in the food industry. The development of adequate flavour and aroma depend on the stringent regulation of important factors such as lipase concentration, pH and temperature of synthesis media and emulsion content (Fallahi et al. 2018).

In the dairy industry, hydrolysis of milk fat is achieved with the help of lipases. Lipases modify the chain length of fatty acids which in turn contributes to the enhancement of flavour and texture of many commercially available cheeses. Lipases are also used to reduce the period of cheese ripening, and they accelerate the lipolysis reaction during the processing of dairy products (butter, milk fat and cream). Animal tissues such as pancreatic glands (bovine and porcine) and pregastric tissues of young ruminants (kid, lamb and calf) are favourable sources for lipase production. The lipases thus produced are used for flavour enhancement in cheese. A new technique of improving flavour involves incubation of cheese with lipases at specific high temperatures to generate enzyme-modified cheese (EMC). These EMCs are intensified with flavour, and they serve as a constituent of other food items (dips, sauces, soups and snacks) (Moskowitz and Noelck 1987). Gastric lipases hasten the ripening process and intensify the flavour of popular cheese varieties like cheddar, provolone and ras (Wilkinson 1993). Introduction of lipase accelerates fatty acid liberation resulting in flavour development. Studies also revealed that when a combination of fungal proteases and lipases was added, cheddar cheese was found to contain highly soluble proteins and free fatty acids. These enzymes improved flavour and shortened the ripening period to 3 months. The concentration of lipase used to accelerate the ripening process needs to be strictly regulated as high enzyme concentration leads to excessive enzymatic reactions resulting in undesired characteristics and yield reduction.

Another major application of lipases is associated with the fat and oil industry. Lipases modify lipid and fat by altering the fatty acid chains of glycerides. This process allows the transformation of a less desirable lipid into a higher value lipid. Thus, lipases catalyse hydrolysis and esterification of oils and fats (Ray 2012; Houde et al. 2004).

Immobilized lipases are used as biosensors for the determination of triacylglycerols. They are used in food industries involved in the production of fats and oils, beverages, soft drinks, pharmaceutical companies and in clinical diagnosis. Samples containing triacylglycerol generate free glycerol in the presence of lipases. The released glycerol units are quantified either by a chemical or an enzymatic method to determine the quality of products (Rejeb et al. 2007). In baking industry, focus is more on lipases as they enhance the flavour content of bakery products by liberating short-chain fatty acids through esterification. Along with flavour enhancement, they also prolong the shelf life of most of the bakery products. Lipase catalysis improves the texture of breads by reducing firmness and increasing the loaf-specific volume, thereby making them softer (Van Oort 2010).

Lipases are a vital constituent of fruit and vegetable juices (Panda et al. 2016). They facilitate the removal of fat from meat and fish products (Gunasekaran and Das 2005). Moreover, addition of lipase to noodles has shown to soften the noodles' texture despite having low levels of the substrate acylglycerols present in the formulations (Suzuki et al. 2010). The immense potential of lipases associated with food and its processing industry should be harnessed to full capacity and can be achieved by developing new cost-effective techniques for increasing its production and

purification. The properties of lipases along with their mode of action need to be well studied and explored to improve their function in extreme conditions as well as to increase their production via genetic engineering. Moreover, novel techniques should also be developed for immobilizing lipases as they are used as biosensors and biocatalysts in food processing technology.

4.3.9 People Networks for Fungal Biotechnology

"The European Fungal (EUROFUNG) network" is a platform to prioritize and advance fungal technologies to aid in fungal biotechnology (Meyer et al. 2016). This network includes academic members, institutions, industries, biotechnological and pharmaceutical companies across Europe. The EUROFUNG network's mission is sustainable bio-economy with human welfare by collaborating across the network and disciplines to accelerate research activity. More policies and networks of such kinds are required to meet food security.

4.3.10 Conclusion and Future Prospects

In conclusion, white fungal biotechnology has potential applications in food and feed industry and is eco-friendly in nature, bringing down greenhouse emissions. The applications include using fungi as food (edible fungi) or fodder and in producing SCPs, processing food (bread, cheese and others) and fermenting food (alcohols and beverages). Fungi are used to enhance flavour in cheeses, bread and beverages; improve protein quality and yield in SCPs; and increase the stability and shelf life of products with much efficacy. Thus, employing fungal white biotechnology meets important challenges like food security (providing food for all). The future of fungal white biotechnology lies in developing fungal strains with improved characteristics, which exhibit tolerance to extreme conditions during processing and the enrichment of products. Omics technologies like genomics, epigenomics, transcriptomics, proteomics, metabolomics, interactomics and phenomics can be implemented and integrated to analyse and understand the traits required by fungal strains to cope up with these extreme conditions. Though there are several initiatives like the EUROFUNG network for a sustainable bio-economy, which encourages white fungal biotechnology, more policies are also required by other countries in the world to meet food security.

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Chapter 5
Volatile Organic Compounds from Endophytic Fungi

Sudipta Roy and Debdulal Banerjee

5.1 Introduction

Volatile organics are ubiquitous in nature. Their distinction lies in their unique physical property of readily diffusing in the atmosphere. Volatile compounds have a low molecular weight and a lower boiling point that facilitate their rapid evaporation or sublimation and create a higher vapor density. Most of these compounds do not readily dissolve in aqueous systems. Volatile compounds include any carbon compound (excepting carbon monoxide, carbon dioxide, carbonic acid, metallic carbides, carbonates, and ammonium carbonate) that may be conjugated with other elements such as hydrogen, oxygen, fluorine, chlorine, or nitrogen. VOCs are released from a range of anthropogenic activities such as burning of fuel (gasoline), wood, coal, or natural gas and may also be emitted from oil and gas fields and as diesel exhaust. Volatiles are released as fumes from solvents, paints, glues, and other products in our daily use.

Interestingly, there are significant biogenic sources for volatile organic compounds (VOCs) also. Most of these biogenic volatiles include isoprene, monoterpenes, sesquiterpenes, and oxygenated compounds such as methanol, hexane derivatives, 2-methyl-3-buten-2-ol, and 6-methyl-5-hepten-2-one. Volatiles of animal or plant origin have been extensively studied in the past whereas microbial volatiles (i.e., bacteria and fungi) have not gained serious attention for years. Moreover, although there is a growing literature on VOCs of bacterial origin with their functional aspects (Schulz and Dickschat 2007; Junker and Tholl 2013; Piechulla and Degenhardt 2014), much less attention has paid to the fungal VOCs (FVOCS) (Bennett et al. 2013; Bitas et al. 2013; Schulz and Dickschat 2007; Piechulla and Degenhardt 2014; Kanchiswamy et al. 2015).

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Approximately 1000 volatile organic compounds (mVOCs) are cited in different reports as produced by 400 different bacteria and fungi so far, among which only 300 VOCs have been characterized from fungi (Chiron and Michelot 2005; Korpi et al. 2009; Lemfack et al. 2014). Interestingly, in the laboratory it has been found that individual fungal species produce a typical pattern of VOCs that may vary depending on growth conditions. The specific profile of volatiles produced by each fungal species is strongly dependent on such environmental factors as temperature, pH, moisture level, nutrients, and age of the culture (Wilkins and Larsen 1995; Bennett et al. 2013; Morath et al. 2012). However, during the past two decades, research on microbial volatile metabolites has significantly intensified. The recent findings about the importance of such volatile metabolites in microbial interactions within fungi or fungi and bacteria and even in the communication between fungi and plants or animals are no doubt very interesting in basic and applied research. Presently, the potential biotechnological applications of such mVOCs are also well illustrated. *Aspergillus*, *Penicillium*, *Alternaria*, *Cladosporium*, *Mucor*, and *Ulocladium* are among the most common VOC-producing fungal genera found in our environment (Bennett and Inamadar 2015). Fungi are capable of producing a plethora of volatile organic compounds (VOCs) belonging to diverse chemical classes, such as terpenoids, straight-chain and branched hydrocarbons, benzene derivatives, naphthalene derivatives, cycloalkanes, alcohols, organic acids, ketones, and aldehydes (Mends et al. 2012; Riyaz-Ul-Hassan et al. 2012; Strobel et al. 2008; Tomsheck et al. 2010), with some special structures.

Molecular approaches such as metagenomics indicate that less than 5% of all fungal species expected to exist on our planet have been characterized thus far (Riyaz-Ul-Hassan et al. 2013). Furthermore, strain variations among the same fungal species suppose them to be highly diverse and suitable for the discovery of new chemical entities, enzymes, and useful volatile organic compounds. The search is now on for novel strains of microorganisms from variegated environments including extreme ecological niches such as geothermal vents, hot springs, the ocean bed, and cold deserts (Foissner 1999). Human beings have been dependent on plants from the very initiation of our civilization, but later gradually we became habituated to synthetic compounds. Surprisingly, after receiving unsatisfying results from synthetic chemical compounds, now we are again in search of natural compounds and here also plants are our savior.

Every plant in this earth harbors a suite of microorganisms which are not harmful to them but on the contrary help their host in many ways. Such classes of microbes are called endophytes. Endophytic microbes are highly diverse and metabolically very sound! Because very little research has been done on endophytic fungi and their volatile organic metabolites, there is a high prospect of finding untold numbers of novel fungal genera existing as plant-associated microbes as well as many novel volatile compounds with significant bioactivity (Strobel and Daisy 2003; Rana et al. 2018a; Rana et al. 2018b; Rana et al. 2016; Suman et al. 2016). Endophytic fungal VOCs are found to induce positive changes in plant growth and vigor, which might be a blessing for agriculture as the demand on agricultural production for fiber, food, and fuel increases exponentially for our ever-growing human population.

Also, at present fungal VOCs (FVOCs) are increasingly being applied in controlling plant pathogens (mycofumigation), in mycodiesel or fuel production and in biosensor production.

Fungal VOCs influence plant growth and defense, interspecies interactions among plants, bacteria, fungi, and nematodes, as attractants of natural enemies, as bio-control agents, and are finding suitable application as pest/insect/herbivore management (Kanchiswamy et al. 2015; Davis et al. 2013; Weise et al. 2014; D'Alessandro et al. 2014). One unique endophytic fungus, *Muscodor vitigenus*, produces sufficient concentration of naphthalene to alter insect behavior (Daisy et al. 2002). This progressive study on endophytic fungal volatile organic compounds (eFVOCs) demonstrates their critical roles in multitrophic interactions and their importance in both the ecosystem and sustainable agriculture systems.

5.2 VOC-Mediated Interaction in Fungal Endophytes

Plant roots are thought to be an important entry point for endophytic fungi: it is assumed that soil fungi enter the plant root tissues through any mechanical incision or abrasion. Roots release a diverse mixture of low and high molecular weight organic compounds that make the root tissues and surrounding environment nutrient rich for a diverse community of microbes (Badri and Vivanco 2009). Soil-borne fungi first colonize at the rhizosphere, then invade the intercellular space, and act as commensals or mutualistic endophytes (Yadav 2018; Yadav et al. 2017; Hardoim et al. 2008; Reinhold-Hurek and Hurek 2011), or dwell within the root tissues as intracellular endosymbionts (Bonfante and Genre 2010; Desbrosses and Stougaard 2011).Volatile organic compounds (VOCs) typically occur as a complex mixture of low molecular weight lipophilic compounds generated at different steps of various metabolic pathways.

The term "volatilome" recently has been proposed to illustrate their structural and functional importance (Maffei et al. 2011). VOCs are responsible for interspecies and intraspecies communication, with involvement in innumerable interactions among plants, antagonists, or mutualistic symbionts in the environment both below and above the ground (Maffei et al. 2010; Wang and Maffei 2011; Garbeva et al. 2014; Lemfack et al. 2014; Kanchiswamy et al. 2015). With their comprehensive inter- and intraspecific interactions, VOCs cause genetic and phenotypic variation in the interacting organisms (Effmert et al. 2012; Piechulla and Degenhardt 2014; Penuelas et al. 2014).

Fungal endophytics are able to detect host plants through a composite array of molecular signaling and initiate their colonization in the very rhizoplane by producing specific plant growth regulating volatile organics (Ortiz-Castro et al. 2009). Additional signals from microbes have a role in plant root morphogenesis. Very recently the role of *N*-acyl homoserine lactone (AHL) has been recognized as a signal molecule in plants altering gene expression in roots; shoots thus modulate defense and cell growth responses (Ortiz-Castro et al. 2009; von Rad et al. 2008).

The endophytic fungus *Gilmaniella* sp. AL12 induced ethylene production in *Atractylodes lancea*, as found in a recent study by Yuan et al. (2016). Pre-treatment of this plantlet with amino oxyacetic acid, that is, an ethylene inhibiter, also suppressed endophytic fungi-induced accumulation of ethylene and sesquiterpenoids. Table 5.1 lists the VOCs produced by various endophytic fungi. The hypotheses generated from this study imply VOCs released by endophytic fungi can provide an important signal mediating the biosynthesis of sesquiterpenoids in *Atractylodes lancea*.

5.3 Bioactivity of Endophytic Fungal Volatiles (eFVOCs)

Fungi are an extraordinarily diverse group of microorganisms that are found in many habitats, even competing with other microorganisms. Endophytes spend a long time in mutual relationships with their host plants. Endophytic association often seems confused with plant pathogens or surface dwellers. So, to verify that a microorganism has an endophytic lifestyle in the true sense, it must should be successfully reintroduced into disinfected seedlings and judged by microscopy, thereby also fulfilling Koch's postulates (Hyde and Soytong 2008). Currently, endophytic fungi have received much recognition for interesting metabolic potential and useful secondary metabolites. From the biotechnological point of view, volatile-producing endophytic fungi exert a broad spectrum of odorous compounds with different physicochemical and biological properties that make them useful in both industry and agriculture (Yuan et al. 2012) (Fig. 5.1).

5.3.1 Fungal Volatiles as Antimicrobial to Plant Pathogens

Fungal endophytes somehow manage habitat adaptation within the plant inner tissues, resulting in improved performance concerning plant protection from various biotic and abiotic stresses. Fungi are considered as a large cell factory for volatiles that can harness organic natural products with possibilities for the development of biocontrol agents. Certain endophytes produce antimicrobial VOCs that directly contribute to plant defense against pathogenic microorganisms. The endophytic fungus *Muscodor albus* can be considered as the most active candidate strain in this context. This strain was first reported as an endophyte from *Cinnamomum zeylanicum* (Worapong et al. 2001). It efficiently inhibits and executes selected other plant pathogenic fungi and bacteria by producing a suite of different volatiles (Strobel et al. 2001) that accounts for at least 28 different volatile organic compounds. Surprisingly, although few individual compounds were antagonistic to certain pathogens, a strong synergistic effect was detected, even to lethality, for a broad range of plant and human pathogenic fungi and bacteria. Derivatives of alcohols, esters, ketones, acids, and fatty acids were found as principal components in the

Table 5.1 List of different volatile organics isolated from endophytic fungi

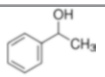
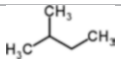
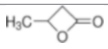
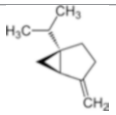
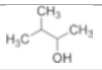
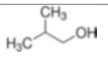
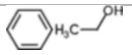
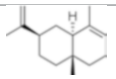
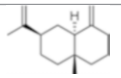
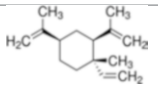
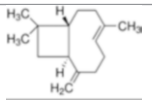
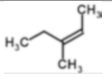
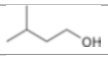
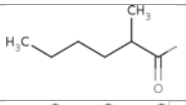
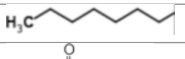
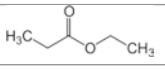
Endophytes	Host plant	eFVOCs	Structure	References
Aspergillus niger	*Rosa damascena*	2-Phenylethanol		Wani et al. (2010)
Botrytis sp. BFT21	*Musa* sp.	2-Methylbutane		Ting et al. (2010)
		β-Butyrolactone		
		2-Butene dinitrile		
Phomopsis sp.	*Odontoglossum* sp.	Sabinene		Singh et al. (2011)
		3-Methylbutan-1-ol		
		2-Methylpropan-1-ol		
		Acetone		
		Benzene ethanol		
Nodulisporium sp.	*Cinnamomum loureirii*	α-Selinene		Park et al. (2010)
		β-Selinene		
		2,5-Dihydrotoluene		
		β-Elemene		
Phialocephala fortinii	Tree root endophyte	β-Caryophyllene		Kramer and Abraham (2012)
Clonostachys rosea (*Gliocaldium roseum*) strain C-13 = NRRL 50072	*Eucryphia cordifolia*	2-Pentene		Griffin et al. (2010)
		3-Methylbutan-1-ol		
		2-Methylhexanoate		
		Heptane		
		Octane		
		2-Butyl propionate		

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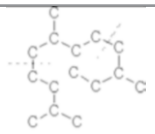
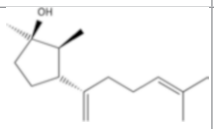
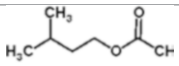
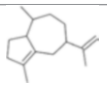
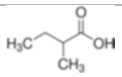
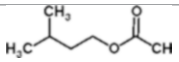
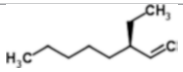
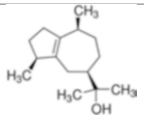
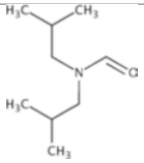
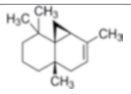
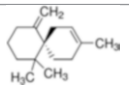
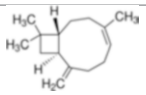
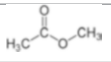
Table 5.1 (continued)

Endophytes	Host plant	eFVOCs	Structure	References
Epichloe typhina	*Phleum pratense*	Sesquiterpenes		Steinebrunner et al. (2008)
		Chokol K		
Muscodor albus	*Cinnamomum zeylanicum*	1-Butanol 3-methyl acetate		Strobel et al. (2001)
Muscodor albus I, 41.3 s	Unidentified tree species	2-Methyl furan		Atmosukarto et al. (2005)
		Aciphyllene		
Muscodor albus E-6	*Guazuma ulmifolia*	2-Methyl-butanoic acid		Strobel et al. (2007)
		3-Methyl butyl ester		
		3-Ethyl 1-octene		
		Guaiol		
		N-(1-Methylpropyl) formamide		
Muscodor sutura	*Prestonia trifidi*	Thujopsene		Kudalkar et al. (2012)
		Chamigrene		
		Isocaryophyllene		
Muscodor albus GBA	*Ginkgo biloba*	3-Methyl acetate		Banerjee et al. (2010)
		1-Butanol		

(continued)

Table 5.1 (continued)

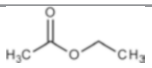
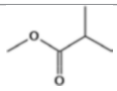
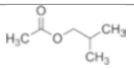
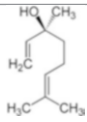
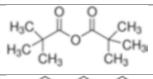
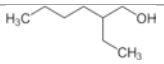
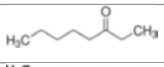
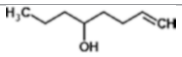
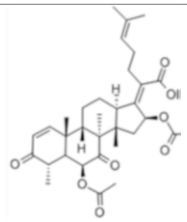
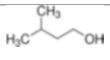
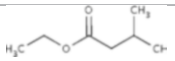
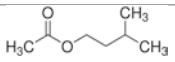
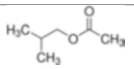
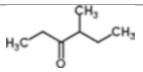
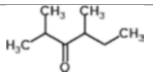
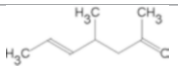
Endophytes	Host plant	eFVOCs	Structure	References
Muscodor albus MOW12	*Piper nigrum*	Acetic acid ethyl ester		Banerjee et al. (2014)
		Propanoic acid 2-methyl-methyl ester		
		Acetic acid 2-Methylpropyl ester		
Daldinia bambusicola	*Camellia caduca*	Linalool		Pandey and Banerjee (2014)
		Pivelic acid anhydride		
		2-Ethylhexanol		
Hypoxylon sp.	*Persea indica*	3-Octanone		Tomsheck et al. (2010)
		7-Octene-4-ol		
Pichia guilliermondii	*Paris polyphylla* var. *yunnanensis*	Helvolic acid		Zhao et al. (2010)
Geotrichum candidum	*Solanum melongena*	3-Methyl-1-butanol		Mookherjee et al. (2018)
		Ethyl 3-methyl butanoate		
		Isopentyl acetate		
		Isobutyl acetate		
Nodulisporium sp. (*Hypoxylon* sp.)	*Thelypteris angustifolia*	4-Methyl-3-hexanone		Riyaz-Ul-Hassan et al. (2013)
		2,4-Dimethyl-3-hexanone		
		4-Methyl 5-hepten 2-one		

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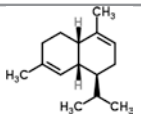
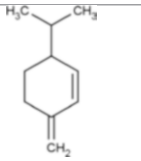
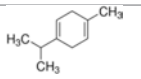
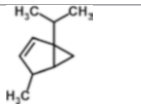
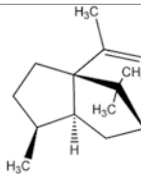
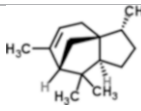
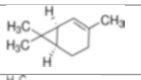
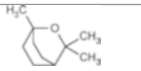
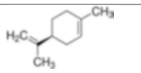
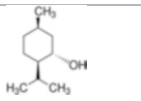
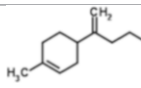
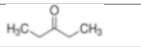
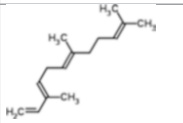
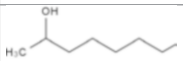
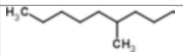
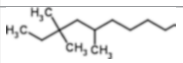
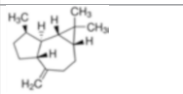
Table 5.1 (continued)

Endophytes	Host plant	eFVOCs	Structure	References
Diaporthe spp.	*Catharanthus roseus*	Muurolene		Yan et al. (2018)
		Phellandrene		
		Terpinene		
		Thujene		
		Patchoulene		
		Cedrene		
		2-Carene		
Nodulisporium sp. GS4d2II1a	*Gliricidia sepium*	Eucaliptol		Sánchez-Fernández et al. (2016)
		Limonene		
Diaporthephaseolorum	*Picrorhiza kurroa*	Isomenthol		Qadri et al. (2015)
		β-Bisabolene		
		3-Pentanone		

(continued)

Table 5.1 (continued)

Endophytes	Host plant	eFVOCs	Structure	References
Gliocladium roseum (NRRL 50072)	*Dicksonia antarctica*	4-Decene		Strobel et al. (2017)
		α-Farnesene		
		Pentyl ester		
Gliocladium roseum (NRRL 50072)	*Eucryphia cordifolia*	Pentyl alcohol		Strobel et al. (2008)
		2-Octyl acohol		
		Undecane 2,6-di-methyl		
		Decane 3,3,5-trimethyl		
		Cyclohexene, 4-methyl		
Muscodor yucatanensis	*Bursera simaruba*	2-Pentyl furan		Macias-Rubalcava et al. (2010)
		Aromadendrene		

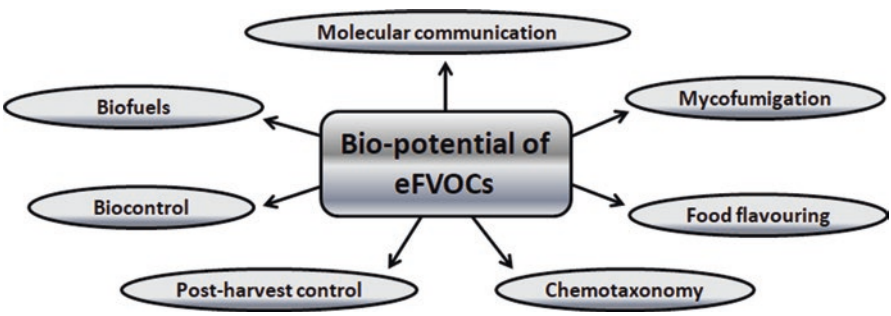


Fig. 5.1 Bioactive potential of volatile organic compounds from endophytic fungi

metabolite mixture of this endophytic strain, among which isoamyl acetate was the most promising biologically active compound (Strobel et al. 2001).

Another endophyte to *Mucor albus* was isolated emitting a number of volatiles, such as tetrohydofuran, aciphyllene, and azulene derivatives (Atmosukarto et al. 2005). The mixture of volatiles effectively inhibited a broad range of plant and human pathogenic bacteria. *Muscodor albus* E-6, an endophyte of *Guazuma ulmifolia*, was isolated by Strobel et al. (2007). This organism produces several unique VOCs that were not previously reported for any other species of *Muscodor*. The volatile metabolites produced by this strain include 2-methyl butanoic acid, 3-methyl butanoic acid, 2-methyl-2-butenal, butanoic acid, 3-methyl butyl ester, 3-methyl-3-buten-1-ol, guaiol, 3-ethyl-1-octene, *N*-(1-methyl propyl) formamide, and certainly azulene with naphthalene derivatives. The mixture is highly effective against an array of plant pathogenic fungi and bacteria.

The endophytic fungus *Muscodor sutura* was observed to produce a mixture of volatile organics including thujopsene, chamigrene, isocaryophyllene, and 2-methyl butanoic acid, which has not been previously reported by any other fungi of the same genus (Kudalkar et al. 2012). This volatile mixture emitted by *M. sutura* inhibited (100% mycelial inhibition) a set of 13 fungal pathogens after only 2 days of exposure. *Muscodor albus* strain GBA was isolated as an endophytic fungus of *Ginkgo biloba*. The strain showed strong inhibitory and killing effects toward test fungal pathogens by its released VOCs. The chemical analysis of VOC revealed derivatives of esters, lipids, alcohols, acids, and ketones with a high concentration of 1-butanol and 3-methyl acetate (Banerjee et al. 2010).

Subsequently, seven new *M. albus* strains were isolated producing various novel mixtures of volatile secondary metabolites. *Muscodor albus* MOW12 (Fig. 5.2), was isolated as an endophytic fungus with antifungal activity from *Piper nigrum* in Mawlong, India. This Xylariaceae-derived endophyte produced low molecular weights of ester, alcohol, and acid derivatives. The major ester components found within a volatile mixture of this isolate are acetic acid ethyl ester, propanoic acid 2-methyl-methyl ester and acetic acid 2-methylpropyl ester (Banerjee et al. 2014). The volatile chemical profile of each *Muscodor* strain significantly varies from one another; even their antagonistic pattern is also remarkable. Some species release large amounts of chemicals as volatile secondary metabolites; all together about 50 different volatiles are documented to be produced by a fungal isolate with endophytic association exerting an impressive antimicrobial spectrum (Ezra et al. 2004). Mycofumigation with *M. albus* against pathogens was already reported in smut-infected barley seeds, and 100% disease control was reported by Strobel et al. (2001). VOC-producing endophytic fungi were also experimentally tested for the treatment of fruits in storage and in transit (Mercer and Jimenez 2004). Soil treatments have also been effectively used in both field and greenhouse situations (Mercier and Manker 2005).

Mitchell and Strobel (2010) isolated *Muscodor crispans*, endophytic to *Ananas ananassoides*. This strain produces a mixture of antifungal and antibacterial volatile organic compounds that strongly inhibits *Pythium ultimum*, a potential plant pathogenic fungus. This strain was also found active against *Alternaria helianthi*, *Botrytis*

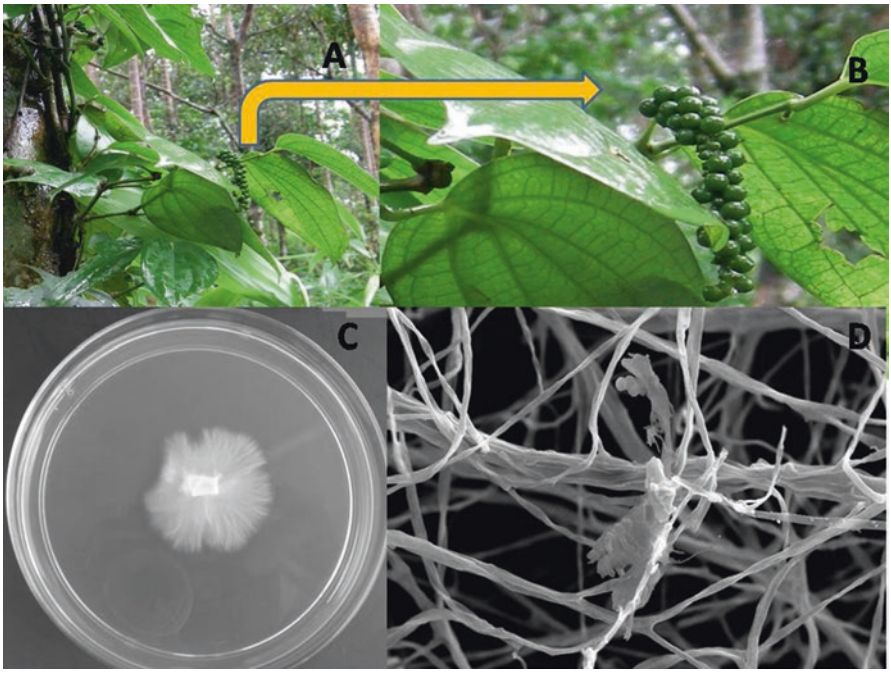


Fig. 5.2 The endophytic strain of *Muscodor albus* MOW12 isolated from *Piper nigrum*: (**a**) the plant, *Piper nigrum*; (**b**) larger view of leaves and fruits of this plant; (**c**) colony on potato dextrose agar media; (**d**) scanning electron microscopy (SEM) observation of 10-day-old culture of *Muscodor albus* MOW12

cinerea, *Fusarium culmorum*, *F. oxysporum*, *Phytophthora cinnamomi*, *P. palmivora*, *Rhizoctonia solani*, *Sclerotinia sclerotiorum,* and *Verticillium dahlia* and even to the plant pathogenic bacterium *Xanthomonas axonopodis* pv. *citri*. The volatile mixture was checked for environmental safety issues and approved as safe by GARS (Generally Regarded as Safe), and no azulene or naphthalene derivatives were detected in the volatile mixture of *Muscodor crispans*, making it a potential candidate strain for commercial application. Thus, this mixture was suggested to have potential utility in applications ranging from food preservation derivatives to agricultural, household, and industrial uses by the US Food and Drug Administration. Stinson et al. (2003) isolated another fungus, *Gliocladium* sp., an endophyte of *Eucryphia cordifolia*, that was discovered to be a volatile antibiotic producer. Many volatile organic compounds were analyzed from the endophytic fungus *Daldinia concentrica*, isolated from olive trees in Israel. This fVOCs mixture was found very promising for post-harvest control. It was effective when used experimentally to protect dried apricots, plums, and raisins from rotting. Moreover, the fVOCs significantly protected peanuts against *Aspergillus niger*, oranges and tomato paste from *Penicillium*, and grapes against *Botrytis*. Another reported endophytic fungus is *Myrothecium inunduatum* from a euphorbeacean herb, *Acalypha indica*, in India (Banerjee et al. 2010).

The authors reported that *M. inundatum* that was cultivated in shake culture flasks produced an abundance of VOCs that showed effective inhibitory effects on the growth of *Phythium ultimum* and *Sclerotinia sclerotiorum*. The volatile mixture contained various hydrocarbons and hydrocarbon derivatives with several terpenes, organic acids, ketones, and alcohols. In another experiment, the volatile antimicrobial metabolites were used to increase the shelf life of fruits and vegetables, as was experimentally shown by Pandey and Banerjee (2014). Endophytic *Daldinia bambusicola* was isolated from *Camellia caduca*. The strain produced linalool, benzene ethanol, and other volatile organics that were able to kill *Phytopthora palmivora* as well as significantly inhibit the growth of *Geotrichium* sp., *Alternaria* sp., *Colletotrichum lagenarium*, *Botrytis cinerea*, and a few others with mild inhibition. The most prevalent compounds were 3-octanone, 3-octanol, and 7-octen-4-ol analyzed, from an endophytic *Hypoxylon* sp. isolated from *Persea indica*, a widespread Laurasian tree of the Mediterranean flora, which produces a plethora of FVOCs with a high effectiveness to treat *Phytophthora cinnamomi*, *P. palmivora*, *Cercospora beticola*, *Aspergillus fumigatus*, and *Sclerotinia sclerotiorum* (Tomsheck et al. 2010). *Pichia guilliermondii* (endophyte to *Paris polyphylla* var. *yunnanensis*) is reported to emit several volatile compounds including helvolic acid. This volatile compound exerts high antifungal activity by inhibiting spore germination of *Magnoporthe oryzae*, one of the most devastating pathogens of rice (Zhao et al. 2010).

Chokol K, another volatile organic compound produced by the grass endophyte *Epichloe* sp. (Clavicipitaceae), effectively inhibited the growth and spore germination of two mycoparasites associated with stomata and two plant pathogenic fungi (Steinebrunner et al. 2008). Endophytic fungi from Orchidaceae were investigated by Singh et al. (2011) for VOCs. *Phomopsis* sp., isolated from *Odontoglosum* sp., produces 3-methyl-1-butanol, 2-methyl-1-propanol, benzene ethanol, and 2-propanone as principal components in the volatile metabolite mixture. Experimental observation indicated that an artificial mixture of these compounds also displayed strong growth inhibition of several fungal pathogens, including *Pythium ultimum*, *Phytophthora palmivora*, *Sclerotinia sclerotiorum*, *Rhizoctonia solani*, *Fusarium solani*, *Botrytis cinerea*, *Colletotrichum lagenarium*, and *Verticillium dahlia*. Endophytic *Botrytis* sp. BTF21, which was isolated from *Musa* sp., was found to produce 2-methyl-butane, β-butyrolactone, and 2-butenedinitrile as volatile secondary metabolites (Ting et al. 2010). The VOC produced by this strain was found to have biocontrol potential to *Fusarium oxysporum*, which is considered to be a serious plant pathogen. *Nodulisporium* sp. and a few more endophytic fungi were isolated from *Cinnamomum loureirii*. As volatile organics, β-elemene, α-selinene, β-selinene, and 2,5-dihydrotoluene were obtained after chemical analysis of the volatile mixture of this *Nodulisporium*. The volatile mixture was successfully applied as a post-harvest disease control of apples (Park et al. 2010).

The volatile sesquiterpene β-caryophyllene was identified to be produce by the endophyte *Phialocephala fortinii* with potential anti-fungal activity (Kramer and Abraham 2012). The VOCs of *Oxyporus latemarginatus* EF069, which was isolated

as an endophyte from red peppers, also had a negative effect on the mycelial growth of several plant pathogens. Mycofumigation with this endophytic fungus was successfully achieved for control of post-harvest apple decay and root rot by *Rhizoctonia* on moth orchid (Lee et al. 2009). *Aspergillus niger* JUBT 3M, isolated from *Rosa damascena*, is also able to produce VOCs. Chemical analysis reveals the production of 2-phenylethanol as a volatile organic by this isolate. The commercial applications of phenyl ethanol include its use in antiseptics, disinfectants, antimicrobials, and preservatives in pharmaceuticals (Wani et al. 2010).

A *Phoma* sp. was isolated and identified as endophytic of *Larrea tridentata*. This fungus produces a unique mixture of VOCs including a series of sesquiterpenoids, some unusual alcohols, and several reduced naphthalene derivatives. Trans-caryophyllene, considered as a product in the fungal VOCs, was also noted in the VOCs of this plant. The volatile mixture produced by *Phoma* sp. exerts strong antifungal effects on *Verticillium*, *Ceratocystis*, *Cercospora*, and *Sclerotinia*. Here it must be noted that this antifungal profile of endophytic isolates is markedly similar to that of the methanolic extract of the host plant (Strobel et al. 2011). Six volatile organic compounds were obtained from two endophytic fungi (*Nodulisporium* sp. strain GS4d2II1 and *Hypoxylon anthochroum* strain Blaci) that were also determined for their bioactivity by Medina-Romero et al. (2017). Results showed that the VOCs have a significant concentration-dependent antifungal effect individually and also act strongly in a synergic manner in both in vivo and in vitro conditions. They also concluded that the mixture of the six compounds may be used for post-harvest control of *F. oxysporum* against tomato wilt. *Geotrichum candidum* was isolated as an endophytic from the fruit *Solanumm elongena*. The volatile mixture produced by this fungus contains 3-methyl-1-butanol, ethyl-3-methylbutanoate, 2-phenylethanol, isopentyl acetate, naphthalene, and isobutyl acetate in significant proportions. The strain showed significant growth inhibition of *Rhizoctonia solani*, a potent plant pathogen. Mild antifungal activity against a few other fungal pathogens was also recorded by this strain. However, the effectiveness of the antimicrobial property of this volatile mixture was enhanced with the exogenous addition of naphthalene (1.0 mg/plate) by Mookherjee et al. (2018).

The volatile composition produced by endophytic fungi *Alternaria alternata* and *Penicillium canescens* (from the leaves of *Olea europaea* L.) displayed a large antifungal spectrum: the six most abundant volatiles were 3-methyl-1-butanol and phenylethyl alcohol (Malhadas et al. 2017). Another *Nodulisporium* species (*Hypoxylon* sp.) has been isolated as an endophyte to *Thelypteris angustifolia*. The endophyte produces VOCs that produce fuel (mycodiesel) and are also used for biological control of plant disease. The organism was responsible for the unique production of a series of ketones, including acetone, 2-pentanone, 4-methyl-3-hexanone, 2,4-dimethyl-3-hexanone, 2-hexanone, and 4-methyl-5-hepten-2-one, with significant concentration in addition to 1-butanol and phenyl ethanol alcohol. The VOCs produced by this strain were found to be lethal to *Phytophthora palmivora*, *Rhizoctonia solani*, *Sclerotinia sclerotiorum*, and *Phytophthora cinnamomi* (Riyaz-Ul-Hassan et al. 2013). *Diaporthe* sp. was isolated as an endophytic fungus from *Catharanthus roseus* (Yan et al. 2018). Identification of its volatile metabolites

showed terpenes including muurolene, phellandrene, terpinene, and thujene, as well as other minor terpenoids such as caryophyllene, patchoulene, cedrene, 2-carene, and thujone. The isolated VOC mixture exhibited significant antifungal properties against a wide range of plant pathogenic test fungi and oomycetes, including *Alternaria alternata*, *Botrytis cinerea*, *Colletotrichum gloeosporioides*, *Fusarium graminearum*, and *Phytophthora cinnamomi*. A total of 70 VOCs were detected, among which mono- and sesquiterpenes, especially eucalyptol and limonene, were a significant fraction from the endophytic *Nodulisporium* sp. GS4d2II1a (Sanchez-Fernandez et al. 2016).

The antagonism assay indicated strong inhibition to oomycetes plant pathogens, including *Pythium aphanidermatum* of economically important crops. Endophytic *Diaporthe phaseolorum* associated with the rhizome of *Picrorhiza kurroa* was evaluated for antimicrobial properties of its volatile metabolites. The strain was found to produce a unique array of VOCs, particularly menthol, phenylethyl alcohol, (+)-isomenthol, β-phellandrene, β-bisabolene, limonene, 3-pentanone, and 1-pentanol. VOCs produced by this strain are selectively active against the growth of plant pathogenic fungi. The role of this endophyte in endophytic association may be to inhibit the growth of pathogens responsible for root rot of the host (Qadri et al. 2014).

In search of VOC-producing endophytic fungi, Strobel et al. (2017) discovered *Urnula* sp., an endophytic fungus of *Dicksonia antarctica*. About 150 different compounds have been detected and identified from the volatile mixture released by this strain by employing carbotrap methodology. The most notable compounds found in the volatile metabolites produced included 4-decene, tridecene, 2-decene, α-farnesene, butanoic acid, and pentyl ester. In vitro assay showed moderate to strong growth inhibition against some common fungal plant pathogens. Although the antimicrobial potential of eFVOCS is well established, only a very few attempts have made to establish its mechanism and for its commercial application.

A very recent article published by Alpha et al. (2015) nicely described the probable mode of action of VOCs produced by *Muscodor albus* CZ-620 through a series of genetic screening and biochemical assays. This experiment suggests that the VOCs produced by this organism may induce alkylation of DNA and ultimately lead to strand breakage in *E. coli*. Additional cytotoxicity profiling indicated that during VOC exposure, *E. coli* became filamentous, with increased cellular membrane permeability. The volatile nature of the toxic compounds produced by *M. albus* and their broad range of inhibition suggest this fungus as an attractive biological agent.

5.3.2 Fungal Volatiles as Diesel Components

The liquid hydrocarbon fuels have high demand worldwide because of the high volumetric density and relative ease of production, transport, and storage (Santos et al. 2014). Many factors are now forcing us to search for alternative sources of liquid

fuels, including issues of the diminishing supplies of these fossil hydrocarbons and serious concerns about climatic changes caused by rising levels of greenhouse gases throughout the world's atmosphere. Plant-derived lipids and bioethanol from the fermentation of sugars and starch are considered an important alternative energy source, but the enormous demand for fossil-based hydrocarbon fuels such as coal, natural gas, and oil cannot be met with the present supply of such alternate energy. Attempts are underway to find still other alternative eco-friendly approaches to increase the production of liquid fuels. Volatile organic compounds (VOCs) are considered to be carbon-based compounds that can readily enter into the gas phase by vaporizing at 0.01 KPa at approximately 20 °C (Pagans et al. 2006).

Most such compounds are lipid soluble and thus have low water solubility. Approximately 250 VOCs have been identified from fungi of diverse ecological niches where they exist as mixtures of simple hydrocarbons, heterocycles, aldehydes, ketones, alcohols, phenols, thioalcohols, thioesters and their derivatives, benzene derivatives, and cyclohexanes (Chiron and Michelot 2005; Korpi et al. 2009; Ortiz-Castro et al. 2009). Recently, endophytic fungi have been extensively studied for production of hydrocarbons and hydrocarbon-like compounds. These compounds have high potential to be used as both "green chemicals" and fuels. *Gliocladium roseum* NRRL 50072, an endophyte isolated from *Eucryphia cordifolia*, produces volatile organics as secondary metabolites (Strobel et al. 2008).

Chemical analysis of the VOC mixture of this fungus revealed an assemblage of alcohols, ketones, and hydrocarbons including pentyl, hexyl, heptyl, octyl, and secondary octyl, decylalcohols, undecane, 2,6,-dimethyl decane, 3,3,5-trimethyl cyclohexene, 4-methyl decane, and 3,3,6-trimethyl undecane. Quantification of the VOCs was determined by proton transfer mass spectrometry (PTR-MS), resulting in organic substances of 80 ppmv (parts per million by volume) in the headspace atmosphere above the media.

The hydrocarbon composition produced by this endophytic fungus contains a number of compounds that are commonly associated with diesel fuel. The mother composition of all types of diesel fuels are the straight-chain hydrocarbons such as hexane, heptane, octane, nonane, and decane along with the branched alkanes, cyclic alkanes, a plethora of benzene derivatives, and some polyaromatic hydrocarbons (Hsu et al. 2000). Currently, all the endophytic fungi producing volatile compounds are being studied for hydrocarbon production or fuel production. An interesting finding by Shaw et al. (2015) states that a nine-carbon polyketide alkene produced by the endophytic fungus *Nigrograna mackinnonii* is likely to be useful for gasoline applications. A great diversity among endophytic fungi is being isolated to date, which implies the occurrence of enormous chemical diversity as invariably moderation of secondary metabolites acts as a weapon in such highly competitive ecosystems (Strobel and Daisy 2003). *Hypoxylon* sp., isolated from *Persea indica*, was found to be an important discovery in this regard: it produces such a volatile organic mixture with fuel properties, that is, mycodiesel.

The VOCs produced by this organism were measured by PTR-MS covering a continuous range of VOC production rate of 7.65 ppmv/h: the VOX mixture consists of 1,8-cineole (a monoterpene), benzene, naphthalene, and 1-methyl-1,4-cyclohexane.

Cineole, known as eucapyptol, can be used in an 8:1 blend with gasoline, whereas this VOC mixture can be used as a diesel fuel additive. The researchers of this group claimed that the ability to produce such rare compounds from a fungal source greatly expands their potential applications in medicine, industry, and energy production. The endophytic *Nodulisporium* sp. also produced some VOC mixtures having both antimicrobial and fuel properties. The presence of cyclohexane, propyl, etc. is considered to be a potential source for a fuel alternative for major diesel components.

The endophytic fungus *Phomopsis* sp. was found to produce 1-butanol, 3-methyl benzene ethanol, 1-propanol, and 2-methyl 2-propanone, which may be used as additives to gasoline. However, extensive basic and applied research is needed to establish hydrocarbon production by these endophytic fungi at the industrial level for additives as liquid biofuel. Such candidate endophytic fungi with fuel potential should be selected for metabolic engineering and scale-up processes for the production of cost-effective alternate fuels that will also be eco-friendly.

5.3.3 Fungal Volatiles as Alleochemicals and Communicating Signals

Allelopathy is a biological phenomenon by which metabolites produced by one organism can influence the germination, growth, survival, and reproduction of other organisms. The biochemicals or metabolites having such properties are called allelochemicals. The term allelopathy was first coined by Hans Molisch in 1937. Alleochemicals are in general a subclass of secondary metabolites produced by plants or microorganisms. The negative role of alleochemicals imparts advantages to plant defenses against herbivores. These biochemicals contribute significantly to species distribution and abundance in plant communities and thus give a constant structure of an ecological niche. Applying such allelochemicals in weed management has become an interesting strategy as an environmentally friendly approach. In the agro-ecosystem, weeds compete with valuable crops for nutrient resources and crop handling that results in reduced crop yield with reduced crop quality and ultimately a huge financial loss every year. Although there are several mechanical and chemical strategies for weed control, resistance to chemical herbicides, change in weed composition, and certainly the potential health hazards of such chemicals forces us to find some alternate strategy to control weeds. In this regard, natural products released from plants or microorganisms are considered as promising alternative options. Allelopathic plant growth inhibition has been demonstrated repeatedly in laboratory-scale experiments, but more realistic field studies involving semi-natural or natural soils are often inconclusive (Inderjit et al. 2005; Macias and Galindo 2007; Kaur et al. 2009).

However, an experiment was well conducted by Macias-Rubalcava et al. (2010) in this regard with *Muscodor yucatanensis*, an endophytic fungus, isolated from the leaves of *Bursera simaruba*. The volatile mixture produced by this endophytic

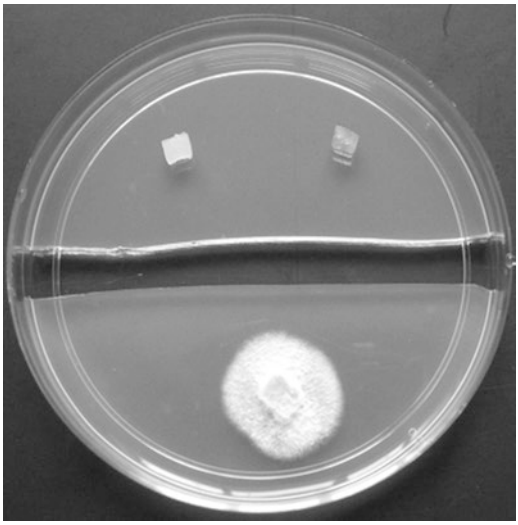


Fig. 5.3 Split plate assay for determination of antifungal activity of VOCs released by endophytic *Muscodor albus* MOW 12 against test pathogens

fungus was analyzed for allelopathic effect on root elongation in amaranth, tomato, and barnyard grass. Significant phytoinhibition was observed by split plate assay (Fig. 5.3) containing potato dextrose agar (PDA) inoculated with *M. yucatanensis* in one compartment. Maximum root growth inhibition was exhibited by a 10-day-old culture of this strain. .Microbes can colonize the surfaces of plant roots, leaves, and flowers in varied proportions, which is dependent on some chemical signaling. In this context volatile fungal metabolites can be considered important signals in the communication of plant-associated fungi in the rhizosphere and endosphere.

5.4 Techniques for VOC Analysis from Endopytic Fungi

Fungi release surplus volatile organic compounds (VOCs) as mixtures of low molecular mass alcohols, aldehydes, esters, terpenoids, thiols, and other small molecules that easily volatilize. Most techniques of VOCs determination including separation and identification now rely on gas chromatography–mass spectrometry (GC-MS). In addition, developments in sensor technology promise to revolutionize this field. Fungal-emitted VOC compositions are complex and highly dynamic. The compounds produced and their abundance also significantly varies with the producing strains, the age of the colony, water availability, substrate utilization type and pattern, incubation temperature, and other environmental parameters.

Endophytic fungi can be isolated from any healthy surface-sterilized plant parts (Strobel and Daisy 2003). There are different authenticated techniques for proper surface sterilization though one can optimize accordingly but authentication of protocol must be done by spreading the last wash water on PDA media to avoid any contamination by surface-dwelling microbes. Different established media are avail-

able for isolation of endophytic fungi. Endophytes should be immediately transferred to sterile media to bring in a pure culture. Before VOC analysis of isolates, the age of strains should be synchronized to obtain uniform and optimum results. For this, the strains may be grown first on PDA or other suitable media in individual Petri dishes, and a culture at least 7 days old can be considered as starter material for the next step of VOC production and analysis. An agar block carefully cut with a sterile scalpel from a full-grown fungal plate can be inoculated and immediately sealed with septa of silicone/plastic tape and threaded cap. After proper incubation the culture vials can be placed in an ethylene glycol bath at about 55–65 °C and the VOCs can be extracted by headspace-solid phase microextraction (HS-SPME) using a polydimethylsiloxane/divinylbenzene (PDMS/ DVB) fiber placed at least 1 cm above the surface of the fungal culture. Then, the fiber can be inserted in the GC-MS system for VOC desorption and chemical analysis of the components (Fig. 5.4). Another automated method of adsorbing and desorbing VOCs accumulated in culture headspace is via SPME, where desorption occurs in the GC injector itself. SPME has gained immense popularity recent years as it allows reduced preparation time while increasing sensitivity over other extraction methods (Zhang and Li 2010). Additionally, headspace-SPME coupled with GC-MS can be employed in direct

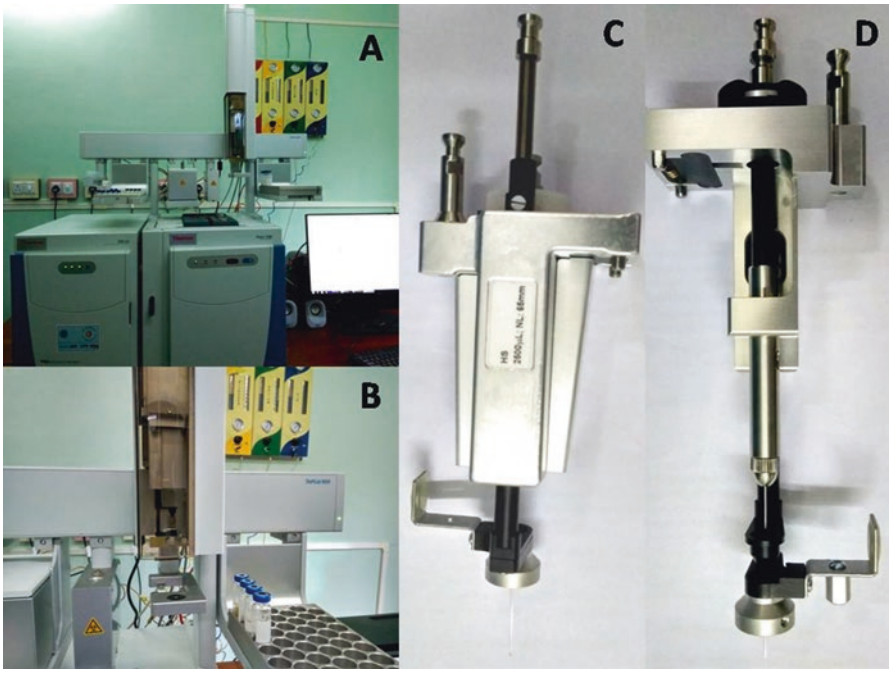


Fig. 5.4 Gas chromatography-mass spectrometry (GC-MS) system, an important device for VOC analysis. (**a**) The instrument. (**b**) Automated solid-phase microextraction (SPME) and headspace system. (**c**) Headspace sample collection device with syringe. (**d**) Headspace-solid-phase microextraction (HS-SPME) syringe

profiling of living fungal cultures (Stoppacher et al. 2010). In another method, the culture headspace can be concentrated using solid adsorbents such as Tenax, followed by thermal desorption into the GC-MS.

Matysik et al. (2009) demonstrated some technical advantages in adsorbing hydrocarbons, esters, ethers, alcohols, ketones, glycol ethers and halogenated hydrocarbons using activated charcoal filters. The VOCs were then desorbed from the activated charcoal pads with 1.5 ml carbon disulfide and introduced into the GC vials for GC-MS analysis. However, less volatile compounds and reactive compounds such as amines, phenols, aldehydes, and unsaturated hydrocarbons were not recovered efficiently from the charcoal bed because they adsorbed strongly to the adsorbing material. This sampling technique combined with GC-MS was applied for the detection of MVOCs emitted by numbers of fungal species in the genera *Penicillium*, *Aspergillus*, and *Cladosporium*. However, the traditional method of simultaneous distillation extraction (SDE) along with vapor distillation and solvent extraction also can be effective for VOC extraction. SDE has been used to examine the VOCs of *Penicillium roqueforti* and compared with SPME (Jelen 2003). However, in an earlier study involving comparative methods analysis for the VOCs of *Penicillium vulpinum* SDE was inadequate to determine a full volatile profile when compared to headspace sampling methods (Larsen and Frisvad 1995).

Selected ion flow tube–mass spectrometry (SIFT-MS) provides rapid and broad-spectrum detection of even trace VOCs in moderately complex gas mixtures. SIFT-MS quantifies VOCs to low part-per-billion levels even in an unmodified atmosphere (i.e., without pre-concentration) in a real-time manner (Senthilmohan et al. 2001). This technique has been used to study VOCs produced by *Aspergillus*, *Candida*, *Mucor*, *Fusarium*, and *Cryptococcus* sp. (Scotter et al. 2005).

Booth et al. (2011) described a technique that rapidly entrap and collects fungal VOCs having fuel potential. The trapping materials, Carbotrap A and B and bentonite-shale, were placed inside a stainless steel column. The trapped fungal VOCs were then recovered via controlled heating of the column followed by passing the eluted gases through a liquid nitrogen trapper. This method allows significantly higher recovery of compounds normally present in the gas phase for bioassays, further separation, and analyses, and potentially for elucidation of structural basis with nuclear magnetic resonance (NMR) spectroscopy to identify novel compounds produced by fungi.

In a separate method, an analytical system was developed by Schoen et al. (2016) for rapid and accurate estimation of total volatile organic compound production from fungal culture. A platinum catalyst and a sensitive CO_2 detector were employed in this system, which determines total VOC production by oxidizing headspace VOCs to CO_2 for detection by the integrated CO_2 detector. Continuous recording of CO_2 data provided a record of respiration and total VOC production throughout the experiments. Respiratory CO_2 was satisfactorily bypassed by the catalyst, and the resultant total VOC content could be easily determined from the difference in the two signals. Finally, proton transfer reaction–mass spectrometry (PTR-MS) was used to identify and measure VOCs. After comparing the sum of the individual

compounds determined by PTR-MS to the total VOCs established with platinum catalyst, potential differences in detection, identification, and calibration can be identified also.

However, in an earlier study, Strobel et al. (2001) employed simpler techniques for analyzing VOCs produced by endophytic *Muscodor albus* from the cinnamon tree. The method analyzed headspace gases of fungi growing on solid media in a Petri dish. A solid-phase micro-extraction syringe was introduced to conveniently trap fungal volatiles. The syringe was placed through a small hole drilled in the side of the Petri plate, exposed to the vapor phase for 45 min, then removed and inserted into a GC-MS system.

5.5 Genetic Engineering with VOC Genes

It is now very clear that microorganisms from diverse ecosystems produce a wide range of volatile organic compounds as secondary metabolites. Compared with other classes of secondary metabolites, volatiles are typically small compounds (up to C-20) with low molecular mass (100–500 daltons), high vapor pressure, low boiling point, and a lipophilic moiety. These properties facilitate their evaporation and diffusion through both water- and gas-filled pores in the rhizosphere and even in the

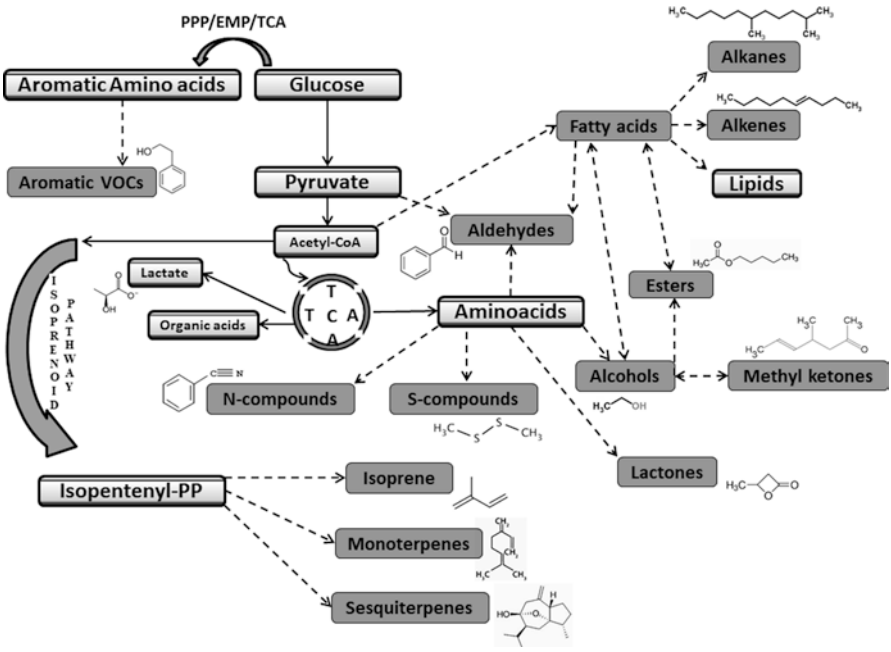


Fig. 5.5 Biosynthetic pathways for FVOCs. ▭ Represents volatile organic metabolites of different chemical nature; – –► line represent the synthetic path for VOCs

physiological systems of the plant. It is notable that fungal volatiles are dominated by alcohols, benzenoids, aldehydes, alkenes, acids, esters, and ketones (Piechulla and Degenhardt 2014), formed mainly by oxidation of glucose from various intermediates (Korpi et al. 2009). The probable biosynthetic pathways for volatile secondary metabolite production are shown in Fig. 5.5. The fundamental biosynthetic pathways are aerobic and heterotrophic carbon metabolism, fermentation, amino acid catabolism, terpenoid biosynthesis, fatty acid catabolism, and sulfur reduction (Penuelas et al. 2014). Various critical factors in the VOC profile and concentration produced by microorganisms include cultural conditions and the physiological status of the producing microorganism (Insam and Seewald 2010; Romoli et al. 2014). A few genes that are involved in VOC synthesis in endophytic fungi have been characterized, opening a new dimension in volatile research and metabolite engineering. It now seems possible to manipulate the quantity and quality of specific VOC production by editing some metabolic pathways. Terpenes are a chemically diverse class of compounds produced as secondary metabolites by many endophytic fungi. These terpenes not only are biologically active secondary metabolites with great pharmaceutical potential but also have potential as an attractive renewable alternative to fossil fuel. As their energy densities are high, different terpenes such as pinene and bisabolene from endophytic fungi are being actively investigated as potential additive biofuels for replacing diesel and aviation fuel. Wu et al. (2016) have isolated and characterized 26 terpene-producing genes (terpene-synthatase, tpr_s) from four mycodiesel-producing endophytic fungi. These tpr_s genes were expressed in an *E. coli* with some modified metabolic pathways to yield an enhanced level of terpene as secondary metabolites. A total of 12 TPR genes among the 26 tested were functional, with most of them exhibiting both monoterpene and sesquiterpene synthase activity.

5.6 Conclusion and Future Prospects

Volatile organic compounds of endophytic fungi have drawn much interest to the present day for their novel structure and potential bioactivity. Most studies have focused on the functional role of volatile organics in plant growth and vigor (Bitas et al. 2013; Penuelas et al. 2014). However, the role of volatiles in fungal and host communication and competition in plant physiological systems is still unclear. Even the specific role of each volatile compound in such endophytic associations is still unknown. It has been proposed that volatiles represent waste material or a detox system for the producing microorganisms (Claeson et al. 2007). A few experiments have established the role of VOCs as info-chemicals to communicate among and between species, in gene expression, and as competitive tools directly exerting antimicrobial activity, thus providing an advantage to the host by suppressing or eliminating potential enemies. Moreover, the interesting point lies in the difference between VOC composition produced by the endophytic fungi on laboratory culture media and that in their original *in planta* environment. Compared with diffusible

compounds, volatile compounds can travel faster and over longer distances through both liquid and gaseous phase systemically in plant tissues, facilitating VOC-based regulation more promptly and stringently. There are studies proving VOCs as signaling molecules, but the intracellular interactions by VOCs at the cellular macromolecular level are still unclear. Future challenges are therefore to find novel chemicals of fungal volatiles, to discover their biosynthetic and regulatory pathways and the genes involved in the biosynthesis of volatiles in endophytic fungi, to determine biologically relevant concentrations, and to resolve the importance of volatiles in ecosystem interactions.

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Chapter 6
Natural Value-Added Compounds from Fungal Communities

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6.1 Introduction

The rapid development of science and technology, as well as industrial processes, have revolutionized the production and utilization of chemically synthesized value-added compounds. Until now chemically synthesized value-added compounds dominated the global markets. However, there is a shift in the research and development of value-added products, and investigations are now focused on alternative strategies to replace chemically synthesized value-added products. Recent reports are suggesting the use of particular metabolic and bio-catalytic properties of microorganisms to potentially transform conventional as well as nonconventional substrates into value-added products. Metabolic products from microorganisms could be an effective alternative for the chemically synthesized value-added products for reasons of their chemical diversity and extended biological activities. Environmental pollutant production during industrial production processes, decreases in substrate selectivity, and the high cost of downstream processes demand microbial production of value-added products (Mishra et al. 2013).

Microorganisms such as bacteria, fungi, and algae are widely studied to isolate value-added metabolic products. Fungal-derived metabolic products are of high importance for their extensive application in the agricultural, food, pharmaceutical, and chemical sectors (Fig. 6.1). These value-added products include drug molecules with antibacterial, antioxidant, and anticancer properties, amino acids, vitamins, organic acid, and industrial chemicals, and biofuels are produced from fungi (Table 6.1). Various technologies are being employed to produce value-added

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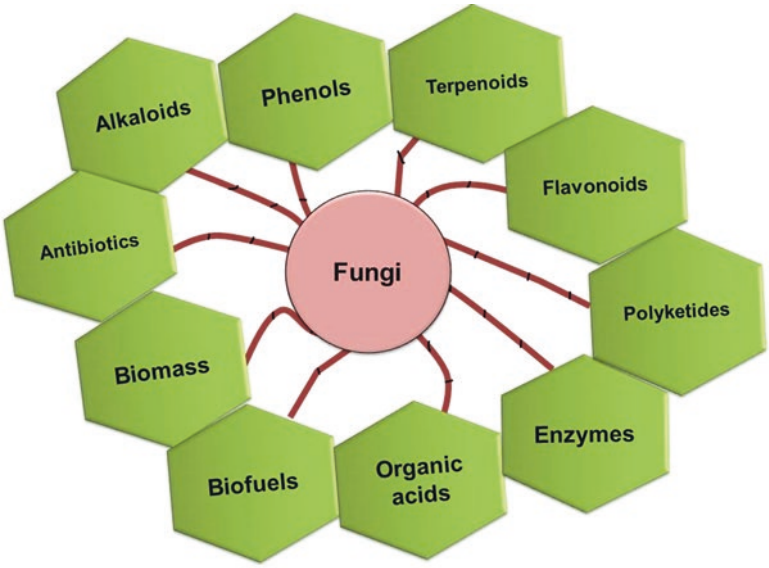

Fig. 6.1 Schematic representation of value-added compounds from the fungal communities

Table 6.1 Some of the important value-added products from fungi and their applications

Value-added products	Fungi	Applications	References
Alkaloids	*Penicillium paxilli, Aspergillus flavus, Emericella desertorum, Neotyphodium lolii, Aspergillus oryzae*	Food, Pharmaceuticals, cosmetics and paper industries	Xu et al. (2014)
Phenols	*Fusarium, Sordariomycetes, Ampelomyces, Alternaria, Aspergillus, Phomopsis, Pestalotiopsis, Phoma, Glomerella, Diaporthe, Verticillium, Nigrospora, Paraconiothyrium, Penicillium, Aspergillus*	Pharmaceuticals, paper and food industries	Pan et al. (2017)
Terpenoids	*Xylaria, Phomopsis, Eutypella, Phyllosticta, Trachelospereum, Artemisia, Tubercularia, Cladosporim, Furarium, Aspergillus, Cladosporium, Arthrinium, Penicillium, Alternaria*	Pharmaceuticals applications	Yan et al. (2018)
Flavonoids	*Alternaria, Fusarium, Schizophyllum, Trametes*	Pharmaceuticals, food, industrial applications	Li et al. (2015)
Polyketides	*Penicillium, Fusarium, Monascus, Alternaria*	Pharmaceuticals, food, industrial applications	Daley et al. (2017)

compounds by chemically synthesis routes (Knez et al. 2017; Paulino et al. 2017). Chemically mediated synthesis of value-added compounds possess major disadvantages such as environmental pollution and health hazards. Moreover, the chemically mediated synthesis of value-added compounds leads to the generation of unwanted by-products that can increase the cost of downstream processes. Fungi utilize various metabolic pathways to produce fungal metabolites for the synthesis of different complex compounds. However, recent advances in science and technology facilitate genetic manipulation technology where potential fungal species can be genetically engineered to improve the yield and productivity of bioactive compounds. This method can even extend to produce novel derivatives from bioactive metabolites (da Silva and Rodrigues 2014; Du et al. 2011; Guedes et al. 2011).

Fungally mediated synthesis of value-added compounds has certain limitations. More than 90% of the potential fungal species cannot be cultivated in laboratory conditions or show comparatively less growth and less metabolite production. These consequences follow the lack of information about the physiological and metabolic behaviours of such fungal species. Only about 5% of the available fungal species are reported for meeting culture conditions in the laboratory. These culturable fungal species also show decline in value-added products in laboratory condition as complete information on their culture conditions is lacking. Hence, steps must be taken to optimize the cultural conditions of the fungal species and enhance the production of metabolites. Advances in genetic engineering provide the space to overcome the mentioned limitations, but sophisticated methods are still required to enhance product yield.

The metagenomics approach is a promising alternative technique to express and isolate metabolic products from uncultivatable fungal species. Genetic engineering procedures can also be employed where a specific gene of interest is isolated from the native producers and incorporated into the genome of other microorganisms to synthesize the compound of interest. To date, *Escherichia coli* and *Saccharomyces cerevisiae* are the organisms most often employed as gene carrier hosts because their physiological and genetic behaviours are fully established, as well as their fast cell growth rate. Advances in biotechnology by protein engineering, metabolic engineering, and synthetic biology led to the discovery of novel biosynthetic pathways and the heterologous expression of metabolic products (Deepika et al. 2016; Du et al. 2011; Gao et al. 2008). This book chapter highlights certain examples of value-added products from fungal species and their potential application in human health. Recent developments in science and technology to improve the yield of metabolic products by strain improvement are also discussed.

6.2 Value-Added Compounds from Fungi

6.2.1 Alkaloids

Alkaloids are the one of the largest classes of nitrogen-containing, low molecular weight secondary metabolites that are found in many organisms including plants, bacteria, fungi, and animals. Alkaloids in the β-carboline group contain compounds

with different pharmacological applications such as antimicrobial, anti-human immunodeficiency virus (HIV), and antiparasitic activities (Patel et al. 2012). Fungi produce chemically diversified alkaloids with significant biological activities. It is well known that most alkaloids are formed by an amino acid decarboxylation reaction. The amino acids tryptophan, tyrosine, ornithine, histidine, and lysine readily participate in the decarboxylation reaction, resulting in the production of alkaloids (Xu et al. 2014). Fungi produce a great diversity of alkaloids with unique molecular complexity in the structure. The available alkaloids are classified into a number of groups based on their native amino acid: morphinane, protoberberine, ergot, pyrrolizidine, and furanoquinoline. Advances in biotechnology led to the identification of the genes that participate in biological synthesis of secondary metabolites (Du et al. 2011).

Alkaloids are isolated from the fungi *Aspergillus*, *Penicillium*, *Pestalotiopsis*, and *Chromocleista*. The maximum number of alkaloids is isolated from species of the genera *Aspergillus* and *Penicillium* in the Fungi kingdom. Alkaloids isolated from a fungal source exhibited significant biological activities including anticancer, antibacterial, antioxidant, antiviral, immunomodulatory, and insecticidal. These wide ranges of biological activities of alkaloids from fungi has focused research to isolate and develop novel broad-spectrum bioactive metabolites with biological applications. Production of alkaloids was identified in the 1940s from *Chaetomium cochliodes*, which led the scientific community to explore diversified fungal species for production of bioactive alkaloids (Ma et al. 2016; Xu et al. 2014).

Although several methods to synthesize the alkaloids by a chemical route are available, certain limitations are caused by the complexity of the molecule. Alkaloids isolated from fungal species are widely accepted and used worldwide because of their broad applicability and therapeutic efficiency with the least adverse effects. Researchers have focused work to isolate alkaloids from fungal communities that have a broad range of biological activity to combat microbial infections. Production of alkaloids from fungi on the industrial scale has certain limitations. Technological advances can overcome these limitations, including reconstitution of the involved biosynthetic pathways and genetic engineering to enhance alkaloids production in significant quantities (Amirkia and Heinrich 2014; Hussain et al. 2018; Patel et al. 2012; Perviz et al. 2016).

6.2.2 Phenols

Phenolic compounds can be defined as chemical substances that most often exhibit an aromatic ring bearing one or more hydroxyl group (OH), including such functional derivatives as ester, methyl ester, and glycosides. Phenolic compounds that are commonly produced as secondary metabolites from fungal species are classified into three groups: simple phenols, phenolic acids, and flavonoids. Most phenolic compounds are derivatives of any one of the following pathways in plants:

pentose phosphate, shikimate, and phenylpropanoid pathways. Interestingly, the plant-associated fungi have symbiotically adopted these pathways into their metabolic cycle and mimic the plants by producing these metabolites. These secondary metabolites are important in plants and fungi by promoting growth and reproduction, providing protection against pathogen and predators. Nevertheless, these phenolic compounds from fungal species exhibit a broad range of physiological properties that include anti-allergenic, anti-artherogenic, antiinflammatory, antimicrobial, antioxidant, antithrombotic, cardioprotective, and vasodilatory activities (Balasundram et al. 2006; Pan et al. 2017).

The endophytic fungi are in symbiotic relationship with plants and produce value-added products as those of plants. Among the several secondary metabolites of fungi, phenolic compounds have gained significant attention for pharmaceutical applications that include antioxidant, cytotoxic, and antimicrobial. The fungal genera *Fusarium, Sordariomycetes, Ampelomyces, Alternaria, Aspergillus, Phomopsis, Pestalotiopsis, Phoma, Glomerella, Diaporthe, Verticillium, Nigrospora, Paraconiothyrium*, *Penicillium*, and *Aspergillus* are the major contributors to phenolic compounds (Wu et al. 2016a; Lunardelli Negreiros de Carvalho et al. 2016; Quang et al. 2018; Rana et al. 2018a, b; Suman et al. 2016).

Microbial phenolic compounds have a significant role in cancer therapy. Unlike other secondary fungal metabolites, phenolic compounds have chemoprotective properties including antioxidant, anti-carcinogenic or anti-mutagenic and antiinflammatory characteristics. These compounds activate the apoptosis process by regulating the cell cycle, regulating carcinogenic metabolisms, arresting DNA binding and cell adhesion, regulating the proliferation and differentiation mechanism of cells, and interfering with the signalling pathways (Huang et al. 2009). In addition to pharmaceutical application of the phenolic compounds from fungal origin, they also actively participate in several other industries such as dairy, food, and cosmetics. For example, phenolic compounds are used in the food industries for the sensory attributes of the food product, and also participate in the development of colour in wine and the addition of flavour and astringency in food products (O'Connell and Fox 2001; Oliveira et al. 2014).

6.2.3 Terpenoids

Terpenoids are naturally occurring hydrocarbons found as secondary metabolites of plants, animals, and microorganisms. Different kinds of terpenoids isolated to date include hemiterpenes, monoterpenes, sesquiterpenes, diterpenes, sesterterpenes, triterpenes, and polyterpenes. Terpenoids are produced by two distinct pathways: the mevalonic pathway and the 2-*C*-methyl-D-erythritol-4-phosphate pathway. In some cases terpenoids are also called iosprenoids, the subclass of the prenyllipids including terpenes, prenylquinones, and sterols. In simple chemical terms terpenoids can

be defined as altered terpenes, wherein a methyl group is substituted or removed, oxygen atoms are removed or added in reactions such as hydrogenation or dehydrogenation, or the carbon backbone is altered either by oxidation reaction or by rearrangements. Terpenoids show various biological activities. For example, the drugs paclitaxel and docetaxel are widely used in cancer chemotherapeutic practice. Terpenoids are also used for microbial infections, hyperglycemia, inflammation, oxidation reaction in the cell, parasitic infections, immunomodulation, and skin permeation enhancement (Ramawat and Mérillon 2013).

An endophytic fungus is a microorganism residing in a plant without causing damage or disease symptoms to the host. In recent years endophytic fungi have gained significant attention as an important source of structurally diverse biologically active compounds with anticancer, antimicrobial, and other biological activities. Some fungal genera such as *Xylaria*, *Phomopsis*, *Eutypella*, *Phyllosticta*, *Trachelospereum*, *Artemisia*, *Tubercularia*, *Cladosporim*, *Furarium*, *Aspergillus*, *Cladosporium*, *Arthrinium*, *Penicillium*, and *Alternaria* are known to produce terpenoids. Sequiterpenes, diterpenoids, and triterpenoids are the common terpenoids produced from endophytic fungi. Terpenoids participate in plant growth and development, and they are also involved in the defence mechanism against insects. The application of terpenoids in plant growth and development was investigated under greenhouse conditions using the terpenoid-producing endophytic fungus *Rhizophagus intraradices*. Test results showed that a plant treated with the endophytic fungus exhibited an enhanced level of production of monoterpenes and sesquiterpenes, which were absent in the control plants. In the same experiment, beet armyworm larvae were allowed to consume the plants treated or not treated with terpenoid-producing endophytic fungi, and the fungal treated plants showed significantly less weight loss compared to uninoculated plants. Terpenoids produced by the fungal species were concluded to have a strong defense response against the beet armyworm (Mousa and Raizada 2013; Pandey et al. 2016; Shrivastava et al. 2015; de Souza et al. 2011; Wu et al. 2016b; Yan et al. 2018; Yu et al. 2010).

Terpenoids, the largest class of secondary metabolites from fungal communities, are employed in the industrial sectors as flavouring agents and fragrances. As mentioned, most terpenoids are biologically active, with therapeutic potential against various human aliments (Singh and Sharma 2015). In recent years, metabolic pathways in the fungi have been altered by genetic engineering to produce significant amounts of terpenoids, enabling the industrial sectors to label the products derived or supplemented with fungal terpenoids as products from natural sources. The synthesis of fungal terpenoid relies on the combination of gene discovery and metabolic engineering that can facilitate higher production. Moreover, as mentioned terpenoids have the greater potential towards plant growth and enhance the defence mechanism against several phytopathogens. Investigations should focus on terpenoid-producing fungal species as biofertilizer to enhance crop productivity (Caputi and Aprea 2011).

6.2.4 Flavonoids

Flavonoids are a large group of polyphenolic compounds with benzo-γ-pyrone structure and are widely produced by plants. Plants produce flavonoids through the phenyl propanoid pathway. Fungal communities present in the host plant promote the defence mechanism in the plants. The adoptive mechanism of the fungal species mimics the metabolic pathways of the plants and produces bioactive metabolites. Endophytes are a group of the fungal community that exhibit mutual relationship with the plants and harbour the similar metabolic pathways of the plants. According to the available reports, several species of endophytic fungi are known for the production of flavonoids. Flavonoids produced from the microbial source possess several pharmacological activities. Researchers reported various beneficial aspects of the flavonoids against different biologicals such as their application in infectious diseases, degenerative diseases, cardiovascular diseases, and cancer and (Jayaprakasha et al. 2005).

Alternaria alternata is an endophytic fungus that potentially synthesizes the flavonoids with significant roles in the plant defence mechanism against phytopathogens. Flavonoids are reported to participate in several plant metabolic pathways including cell signalling, plant growth, and reproduction. Certain flavone derivatives possessing *ortho*-hydroxyl groups on the β-ring gained significant interest for their capacity to detoxify reactive oxygen species (ROS) and promote siderophore production; these specular behaviours aid in the plant defence mechanism (Garrido-Arandia et al. 2016). Interestingly, flavonoids are involved in the signalling mechanism for plant–microbe interactions where they are important in regulating symbiosis in the pre-symbiotic phase. The role and effect of flavonoids vary in different stages of fungal development including pre-symbiotic, spore germination, hyphal length, and differentiation stages. To prove these mentioned properties of flavonoids, the effects of flavonoids with each developmental stage of the four tested fungal species *Gigaspora rosea, Gigaspora margarita, Glomus mosseae*, and *Glomus intraradices* (*Rhizophagus irregularis*) were investigated. Results showed that the effects of flavonoids differed among fungal species in the pre-symbiotic stage (Scervino et al. 2005).

Qiu and coworkers successfully isolated and identified two flavonoid-producing endophytic fungi from *Ginkgo biloba* L. Herein, two fungal isolates, namely, *Aspergillus nidulans* and *Aspergillus oryzae*, were isolated from *Ginkgo biloba* L. Reporting of the production of flavonoids suggested that these two fungal isolates can be employed for the development of natural medicines and prodrugs (Qiu et al. 2010). Some fungal genera including *Alternaria*, *Fusarium*, *Schizophyllum*, and *Trametes* are also known to produce flavonoids, which are involved in the protection of plants from UV radiation, pigment production, induce the synthesis of defence compounds against plant pathogens, and facilitate the signalling mechanisms involved in the symbiotic relationship between plant and fungal species (Hassan and Mathesius 2012; Li et al. 2015).

6.2.5 Polyketides

A wide variety of biologically active natural compounds are produced from fungal species. Among the natural bioactive compounds, polyketides are secondary metabolites produced from fungi with structurally diverse groups. The biosynthetic processes involved in the production of polyketides are highly programmed. The fungal polyketides are synthesized by type I polyketide synthases through a condensation reaction between acetyl-coenzyme A and malonyl-CoA. Type I polyketide synthases have a major role in the formation of the carbon skeleton of the polyketides, which are formed with single modular ornamentation with ketosynthase, acyl transferase, and acyl carrier protein domains (Fujii 2010). Polyketides are a widely used class of secondary metabolite of fungi as fungal polyketides have a broad spectrum of biological activity. For example, griseofulvin, penicillic acid, mycophenolic acid, strobilurins, lavastatin, and squalestatins are antifungal, a powerful antibiotic with toxic effect in clinical studies, immunosuppressive agents, and antifungal agents involved in the control of cholesterol level and heart-associated diseases and an effective inhibitor of squalene synthase, respectively (Kakule et al. 2014). The polyketides are synthesized by the fungal genera *Penicillium*, *Fusarium*, and *Alternaria*. The polyketide lavastain is mostly produced by the fungal genera *Monascus* and *Aspergillus* (Daley et al. 2017).

Neofusicoccum parvum was investigated for the production of polyketides. Four naphthalenone polyketides were isolated from the fungal culture with significant biological activity (Burruano et al. 2016). Wang and coworkers investigated the diversity of the culturable endophytic fungi from Dongxiang wild rice and detected β-ketasynthase in the gene clusters of polyketide synthase. Among the fungal isolates, 13 fungal strains showed antagonistic activity against phytopathogens, and 9 fungal strains had the polyketide synthase gene cluster in their genome, indicating their ability to synthesize polyketides (Wang et al. 2015). The structural complexity of polyketides, which possess multiple stereocenters and numerous oxygen-containing substituents, complicates their synthesis by a chemical route. This limitation can be overcome by screening the microorganisms that produce novel polyketides by molecular tools. The metabolic pathways of these organisms can be genetically engineered to yield the polyketides in significant quantities (Bond et al. 2016).

6.2.6 Enzymes

Enzymes are the most explored and utilized metabolites from microbial sources as they are important in industries for processing the substrate and raw materials. Fungal enzymes function as bio-catalysts to enhancebio-processing reactions in an environmentally friendly and cost-effective manner, unlike chemical catalysts. The microbial enzymes showed several special characteristic features attractive to industry for commercial application, including thermotolerance, thermophilic nature,

tolerance to wide range of pH, and stability of the enzyme activity over a broad range of temperature and other adverse conditions. These enzymes are potentially employed in different industrial sectors (Fig. 6.2) including food, leather, textiles, and animal feed, and in bioconversion and bioremediation (Nigam 2013). Most of the commercially employed enzymes are recovered from a fungal origin; among these, hydrolytic depolymerases are important. Such enzymes as protease, amylases, cellulose, xylanase, lipase, and phytase are most commonly used for industrial applications (Yadav et al. 2016, 2017a, b). *Aspergillus*, *Rhizopus*, and *Penicillium* are utilised widely to isolate commercially important enzymes (McKelvey and Murphy 2011) (Table 6.2).

6.2.6.1 Proteases

Certain groups of fungi secrete the protease enzymes extracellularly to degrade available protein in the environment for their growth, especially saprophytic and pathogenic species (Yike 2011). The protease enzyme hydrolyses the peptide bonds of the proteins and converts them into small peptides and amino acids. Fungal proteases have significant advantages in fermentation technology, unlike chemical

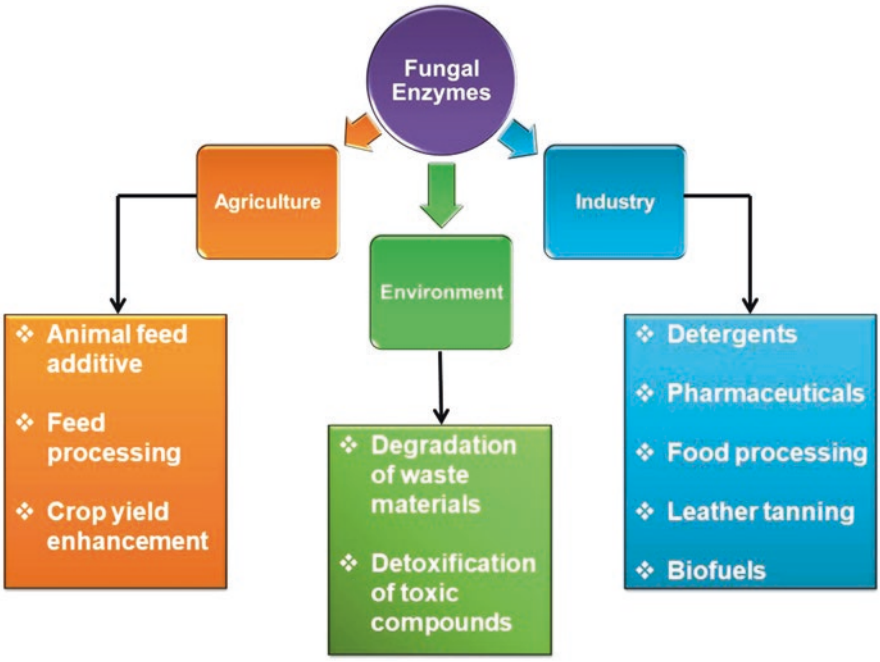

Fig. 6.2 Schematic representation of application of fungal enzymes in agriculture, environment, and industry

Table 6.2 Some important industrial enzymes produced by fungi and their applications

Enzyme	Fungi	Applications	References
Proteases	*Graphium putredinis, Trichoderma harzianum, Aspergillus flavus, Aspergillus niger, Aspergillus fumigatus, Penicillum* sp.	Food, detergents, pharmaceuticals, leathers, textiles, cosmetics and paper industries	Savitha et al. (2011), Chandrasekaran et al. (2015)
Amylases	*Cylindrocephalum* sp., *Aspergillus* sp., *Emericella* sp., *Mucor* sp., *Mycosphaerella* sp., *Penicillium* sp., *Rhizopus* sp.	Starch, paper, food, pharmaceutical industries	Sunitha et al. (2012), Saleem and Ebrahim (2014)
Cellulases	*Trichoderma* sp., *Penicillium* sp., *Botrytis* sp., *Aspergillus* sp., *Rhizopus* sp., *Fusarium* sp.	Production of alcohol from celluloses	Imran et al. (2016)
Xylanases	*Trichoderma* sp., *Aspergillus* sp., *Fusarium* sp., *Pichia* sp.	Biomass processing, paper and textile industries	Sakthiselvan et al. (2014)
Lipases	*Aspergillus* sp., *Mucor* sp., *Penicillium* sp., *Trichoderma* sp., *Colletotrichum* sp.	Food, detergents, pharmaceuticals, leathers, textiles, cosmetics and paper industries	Geoffry and Achur (2018)
Laccase	*Aspergillus flavus, Coriolus versicolor, Streptomyces cyaneus, Phanerochaete chrysosporium, Schizophyllum commune, Pycnoporus cinnabarinus*	Food processing industry, decolourization, detoxification, azo dyes, medical and health care	Pooja et al. (2016)
Nitrilases	*A. furmigatus, F. oxysporum, A. niger, F. solani*	Carboxylic acids production, waste treatment and surface modification	Gong et al. (2012)
Hydrolase	*Geomyces* sp., *Glomerella* sp., *Pseudeurotium* sp., *Gnaphosa fallax*	Food, detergents, pharmaceuticals, leathers, textiles, cosmetics and paper industries	Krishnan et al. (2016)
Pectinase	*Aspergillus* sp., *Rhizopus* sp., *Penicillium* sp.	Food, pharmaceuticals, and cosmetics industries	Barman et al. (2014)
Phytases	*Aspergillus ficuum, A. oryzae, A. amstelodami, A. candidus, A. flavus, A. repens*	Animal food industries	Gupta et al. (2014)

catalysts, which are expensive and have complex downstream processes (Chandrasekaran and Sathiyabama 2014; Souza et al. 2015). Two fungal species, *Graphium putredinis* and *Trichoderma harzianum*, showed higher production of protease enzyme in soya bean meal, and their application was investigated to promote the wash performance of detergents. The isolated protease was stable at a wide range of physiological conditions, exhibiting high stability and reactivity even after the addition of EDTA and sodium perborate. The products of protease are required in detergent-related industries to enhance the efficacy of detergent performance for

the effective removal of various stains (Savitha et al. 2011). Similarly, work has been conducted on paddy soil for the isolation of filamentous fungi and investigating the production of protease enzyme. Culture conditions were optimized for the maximum production of the protease enzyme. The isolated fungi *Aspergillus flavus*, *Aspergillus niger*, *Aspergillus fumigatus*, and *Penicillium* sp. produced the protease enzyme (Chandrasekaran et al. 2015).

6.2.6.2 Amylases

Aamylases are utilized in the degradation of starch and have significant importance in industrial sectors with economic benefits. Different classes of amylases are α-amylase, β-amylase, and glucoamylases, where α-amylases are involved in breaking the 1,4-α-D-glycosidic linkage between adjacent glucose units in the linear amylase chain; β-amylase breaks down the nonreducing ends of amylose, amylopectin, and glycogen molecules; and glucoamylase cleaves the single glucose units from the nonreducing ends of amylose and amylopectin (Singh et al. 2014). The fungal amylases have potential wide application in the food and pharmaceutical industries. Herein, a study has been conducted to isolate the amylase-producing fungus *Cylindrocephalum* sp. from the plant *Alpinia calcarata*. The fungal isolate showed maximum amylolytic activity under a wide range of physical and chemical conditions including pH, temperature, carbon, and nitrogen source (Sunitha et al. 2012). Fungal genera such as *Aspergillus*, *Emericella*, *Mucor*, *Mycosphaerella*, *Penicillium*, and *Rhizopus* are utilized for the production of amylases (Saleem and Ebrahim 2014).

6.2.6.3 Cellulases

Cellulase are useful in such applications as the energy production, pulp, paper, textile, and animal feed industries. Cellulase is also used extensively in food industries such as bakeries and in the production of wine and fruit and vegetable juice (Yopi et al. 2017). Cellulose is the most abundant biopolymer available on Earth and its hydrolysis results in the production of sugars, sugar acids, and phenolic substances. Cellulosic substrates contribute to global energy, chemical, and material demands in a renewable and sustainable manner. Recent research reported that more than one fourth of the current use of petroleum products will be replaced by the fuels generated by biomass conversion with the help of the enzyme cellulase (Payne et al. 2015). Some fungal communities such as *Trichoderma*, *Penicillium*, *Botrytis*, *Aspergillus*, *Rhizopus*, and *Fusarium* are potential producers of cellulase enzyme (Imran et al. 2016). A study to isolate the cellulase-producing filamentous fungi from soil samples and evaluate hydrolytic activities found that two filamentous fungi, namely, *Trichoderma* and *Aspergillus*, were isolated with significantly higher production of cellulase enzyme (Ja'afaru 2013).

6.2.6.4 Xylanases

Xylanases have several industrial applications for the preparation of food, feed, pharmaceuticals, and also ingredients for the paper and pulp industries. Filamentous fungi have gained significant attention for the production of xylanases on an industrial scale. Moreover, the enzyme xylanase produced by filamentous fugal communities was in significantly higher concentrations than other producers such as bacteria and yeast. Fungal genera such as *Trichoderma*, *Aspergillus*, and *Fusarium* are potential producers of xylanases (Sakthiselvan et al. 2014),which recently attracted attention for biotechnological application in the production of xylitol and ethanol. Xylanases are used extensively to convert agricultural waste that contains cellulose and hemicellulose into sugars. Xylanases are also used in the textile industry to process plant fibres (Liao et al. 2015).

6.2.6.5 Lipases

Lipases catalyse the hydrolysis of long-chain triglycerides to glycerol and free fatty acid. The special feature of lipase for chemical transformations has increased its demand in the food, detergent, chemical, and pharmaceutical industries. Soils contaminated with oils, vegetable oils, petroleum refinery waste, and dairy industry waste harbour lipase-producing microorganisms (Singh and Mukhopadhyay 2012). *Aspergillus*, *Mucor*, *Penicillium*, *Trichoderma*, and *Colletotrichum* are known for significant lipase production (Geoffry and Achur 2018). Griebeler and coworkers isolated the fungal genera *Aspergillus* and *Penicillium* from the soil to evaluate their production of lipase enzyme. Both these fungal genera are potential producers of lipase enzyme with different genetic identity (Griebeler et al. 2011). Fungal lipases have significant potential for use in industrial processes because of their stability in different adverse conditions of pH, temperature, and organic solvents (Pereira et al. 2014).

6.3 Application of Fungi in Biofuel Production

Biofuels have gained considerable attention worldwide as renewable energy to replace petroleum products in the near future. Biofuel advantages include, as compared to petrodiesel, reduced exhaust emission, greater cetane number, biodegradability, lack of sulfur, inherent lubricity, positive energy balance, and higher flash points, compatibility with existing fuel engines, renewability, and domestic origin (Tabatabaei et al. 2015). With currently available technology, the production of biofuel encounters certain limitations in that most of the biofuel is produced using a feedstock as substrate. The common feedstocks used for biofuel production are edible oil crops such as rapeseed, palm, soybean, and sunflower. Hence, current research on biofuels is focused on technology to produce biofuel using cheaper and

nonedible substrates. Fungal cellulases are extensively studied for the hydrolysis of cellulose polymers to monomeric fermentable sugars to produce biofuels. Cellulases are in significant demand in commercial biofuel production industries (Srivastava et al. 2018). The filamentous fungus *Mucor circinelloides* was studied for the efficient production of biofuel as it produces a higher amount of saponifiable matter and has a suitable fatty acid profile for the effective production of biofuel synthesized by acid-catalysed transesterification of extracted microbial lipids and direct transformation of dry microbial biomass. This method showed higher biofuel production with increased FAME yield, suggesting biodiesel production by direct transformation of fungal biomass without an intermediate lipid extraction step (Vicente et al. 2009). A similar study was conducted with the isolated filamentous fungus *Aspergillus* sp. employed as whole-cell biocatalyst for biofuel production using Sabourauds dextrose broth medium and corncob waste liquor. The growth medium enhanced biomass and lipid production with time. Furthermore, fungi cultured in the medium with added substrate showed significant increase in biomass, lipid production, and substrate degradation. Biofuel produced by the fungal isolate was in compliance with biofuel standards. The biofuel obtained in this study is cost effective and can be an alternative to petroleum products in the future (Venkata Subhash and Venkata Mohan 2011).

6.4 Conclusion and Future Perspectives

The bioactive metabolites isolated from fungi have significant impact in the pharmaceutical and biomedical sectors. However, more promising technologies are needed to exploit the fungal communities to produce novel bioactive compounds. Although several biologically active fungal metabolites have been identified and characterized, most of them have only been studied for a few biological activities. Furthermore, some of the fungi reported for the production of bioactive compounds failed to sporulate using non-culture techniques for these unculturable fungi. Recent advances in biotechnology made it possible by genetically engineering the bioactive metabolite-producing fungi. So far the scientific community has focused on the discovery of novel biologically active compounds from culturable fungal communities. However, it is important to explore the non-culturable fungal communities using metagenomic approaches. Certain unknown mechanisms are acquired by fungi from their host to produce metabolites. Interestingly, some of the fungal species are able to produce bioactive metabolites which are not produced by the native host. These exceptional behaviours of the fungi require investigation to understand the molecular mechanisms, involved in the production of bioactive compounds and to provide knowledge about host–microbe interaction.

The fungal communities are the potential source of several value-added compounds. Currently, demand for natural products is rising dramatically as an alternative to developing chemically synthesized products. Hence, there is urgent need to investigate potential sources for the production of value-added compounds. Fungi

are one of the major sources or producers of value-added products in significant quantities. Several bioactive compounds from fungi are reported with desired pharmaceutical applications including antimicrobial, antioxidant, antiinflammatory, and anticancer. Fungi also produce various insecticidal and nematicidal compounds for agricultural application. The bioactive compounds from fungal communities are not only used for human application but also aid plants and animals by promoting growth and development and providing protection from pathogens and invaders. Advances in science and technology have accelerated research on fungal communities as treasures of biologically active and novel value-added compounds.

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Chapter 7
Natural Product Synthesis by Fungi: Recent Trends and Future Prospects

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7.1 Introduction

Natural products are normally resulting secondary metabolites or are produced from microorganisms, plants, or animals (Demain and Adrio 2008; Luckner 2013; Razzaghi-Abyaneh and Rai 2013; Seyedjavadi et al. 2019), continuing to be an incredible resource for the discovery of new drugs (Li and Vederas 2009; Challinor and Bode 2015). Soil microbes remain the most popular sources of natural products for pharmaceutical investigation and development. However, it is becoming very hard to detect new microbial metabolites after more than 60 years of investigations focused on soil microbes, particularly members of the genus *Streptomyces*, from which many antibiotics and other bioactive secondary metabolites with unique pharmacophores have been discovered (Knight et al. 2003; Monciardini et al. 2014). To avoid rediscovery of known compounds from microbes, many approaches are employed to obtain high-quality isolates and novel microbes, with various studies focused on poorly investigated extreme biological habitats (Lam 2007). In the past 20 years, investigations on secondary bioactive metabolites from fungi have advanced and various compounds with antimicrobial, insecticidal, cytotoxic, and anticancer and other activities were discovered from fungi. These compounds were structurally classified as alkaloids, lactones, phenols, quinines, terpenoids, steroids,

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and lignans (Bano et al. 2016; Lobo et al. 2018). Therefore, natural products from fungi represent a diverse potential source of new products for medicine, agriculture, biotechnology, and nanotechnology (Aly et al. 2010; Abdelmohsen et al. 2017).

Fungi belong to a group of microbes known as microorganisms. A characteristic feature of many fungi is their ability to produce secondary metabolites or small molecule natural products. Microbial secondary metabolites include nearly 50,000 known compounds with an extremely diverse array of chemical structures. Fungi, a large group of organisms with a wide variety of sizes and shapes (Gunatilaka and Wijeratne 2000), are everywhere in every environment of the world, growing in soil, acidic hot springs, radioactive wastes, water, and deep in the Earth's crust, as well as in organic matter and the living tissues of most organisms including plants and animals (Rana et al. 2016, 2018; Saxena et al. 2016; Suman et al. 2016; Gunatilaka and Wijeratne 2000). Fungi are used in the manufacture of chemicals and also in the drug development industry. Fungi are eukaryotic organisms known to inhabit almost all the environmental niches of the world and have the capability to develop different solid substrates as a consequence of their varied biological and biochemical evolution. The fungal kingdom contains some of the most important organisms, in both ecological and economic terms, including the well-known fungi such as mushrooms, rusts, smuts, puffballs, truffles, morels, molds, and yeasts. Fungi contribute to the nutrient cycle of ecosystems by breaking down dead organic matter (Gunatilaka and Wijeratne 2000; Schueffler and Anke 2014). Fungi are known to cause a number of plant and animal diseases. In humans, ringworm, athlete's foot, and several other diseases are initiated by fungi. Various fungi are prolific sources of secondary metabolites. Soon after World War I, the British researcher, Harold Rainstrick, began the first systematic study of fungal metabolites. He and his team made seminal contributions to the recognition o f fungi as a major source of natural products.

More than 1,000,000 species of fungi are believed to exist all over the Earth, of which only approximately 70,000 species (less than 5%) have been discovered and reported so far (Schueffler and Anke 2014). The morphological forms range from microscopic unicellular yeasts to multicellular macroscopic fungi. The vegetative structure of most fungi contains thin-walled, branched, transparent, or unbranched hyphae. In many simple fungi (especially yeasts and chytrids), the vegetative structure consists of a single microscopic cell, ellipsoidal, spherical, irregular, or tubular in shape. However, the uniqueness of the fungi lies in (a) their capability to create a surprisingly large diversity of enzymes (conferring on them the ability to colonize and reduce a great variety of substrates), and (b) their potential to synthesize an amazing variety of metabolites with their biological activity. Moreover, the hyphae present a large surface area through which the fungi can interchange substances with the environment, absorbing vital materials required for growth and development and excreting the waste products. Technology based on the degradative or synthetic activities of the fungi has become an integral part of human society and, hence, of our commercial setup as well. Current commercial products of the fungi include amino acids, antibiotics, alcoholic beverages (including distilled alcohol),

fuel (ethanol, biogas), biopesticides, mycoherbicides, bread, cheeses, fermented foods, foods (mushrooms, etc.), single-celled protein, flavours, food colourants, preservatives, soy sauce, vitamins, organic acids, and mycelial paper. Bioremediation, ensilage, biotransformation and many such processes involve the utilization of the fungi. In the emerging 'age of biotechnology,' the fungi are expected to provide a wider range of useful products and processes for human welfare under the banner of what is called fungal biotechnology. Applications of fungal activities already dominate present-day biotechnology (Nisa et al. 2015; McKelvey and Murphy 2017).

In recent years, studies of fungal metabolites have experienced a tremendous increase in response to the need for compounds having biological activity with possible pharmaceutical and agricultural applications (Gunatilaka and Wijeratne 2000; Sexton and Howlett 2006). Antibiotic, antifungal, immunosuppressive, and cholesterol-lowering agents derived from fungi have been used in clinics during the past five decades, contributing significantly to the welfare of mankind and to the spectacular increase in life expectancy observed in the second half of the twentieth century. Soil-borne, parasitic, and saprophytic fungal sources are relatively well investigated for their secondary metabolites. However, interest in secondary metabolites of symbiotic fungi that live in association with terrestrial plants, lichens, marine organisms, and insects has recently intensified because natural substances produced by these fungi for their ecological interactions, especially with their hosts, are expected to exhibit biological activities (Gunatilaka and Wijeratne 2000; Coleman et al. 2011). Studies in mold biochemistry led to the recognition of other fungal products (galic acid, gluconic acid, itaconic acid, glycerol, enzymes, antibiotics) and industries based on these products followed. Figure 7.1 illustrates some important fungal metabolites and their biological effects. Fungi are also sources of commercial chemicals, such as citric and gluconic acids (*Aspergillus niger*), vitamins (riboflavin from *Eremothecium ashbyii*), polysaccharides (from *Aureobasidium pullulans*), and enzymes (rennin from *Rhizomucor pusillus*; lipase from *Penicillium roquefortii*; protease from *Aspergillus oryzae*; cellulase from *Trichoderma viride*). A wide variety of biochemical conversions and modifications of molecules such as sterols can also be processed by fungi (Roukas 2000; Pujari and Chandra 2000; Viniegra-González et al. 2003).

The discovery and development of penicillin in particular provided a great thrust to research activities concerning fungi, especially from an industrial point of view. Extensive details of these investigations are given by Campbell (1983), Ligon (2004), and Muñiz et al. (2007). Most of the world's penicillin today is derived from *Penicillium chrysogenum*. Although hundreds of fungi have been found to show antibiotic activity, very few have found wider application, although cephalosporins (*Cephalosporium acremonium*) and griseofulvin (*Penicillium patulum*) are manufactured in large quantities. Medicinal fungi have been identified as remarkable therapeutic agents in traditional folk medicine and to be important as popular culinary products the world over (Gargano et al. 2017). Species of medicinal mushrooms have a long history of use for disease treatment in folk medicine, especially in countries such as China, India, Japan, Mexico, and Korea (Yang and Jong 1989;

Fig. 7.1 Classification and biological activity of fungal natural products

Park and Lee 1999; Guzmán 2008). Fungi have shown therapeutic action against the development of many diseases, primarily because they contain a number of biologically active compounds (Valverde et al. 2015). Fungi are also used in cosmetics because of their medicinal properties (Chaturvedi et al. 2018). Moreover, they are used in agriculture, including mainly high molecular weight compounds such as polysaccharides, proteins, and lipids as well as a number of low molecular weight metabolites such as lectins, lactones, terpenoids, alkaloids, sterols, and phenolic substances (Brakhage and Schroeckh 2011). Fungi provide a diversity of new secondary bioactive metabolites with unique structure, produced via different biosynthetic pathways (Fig. 7.2).

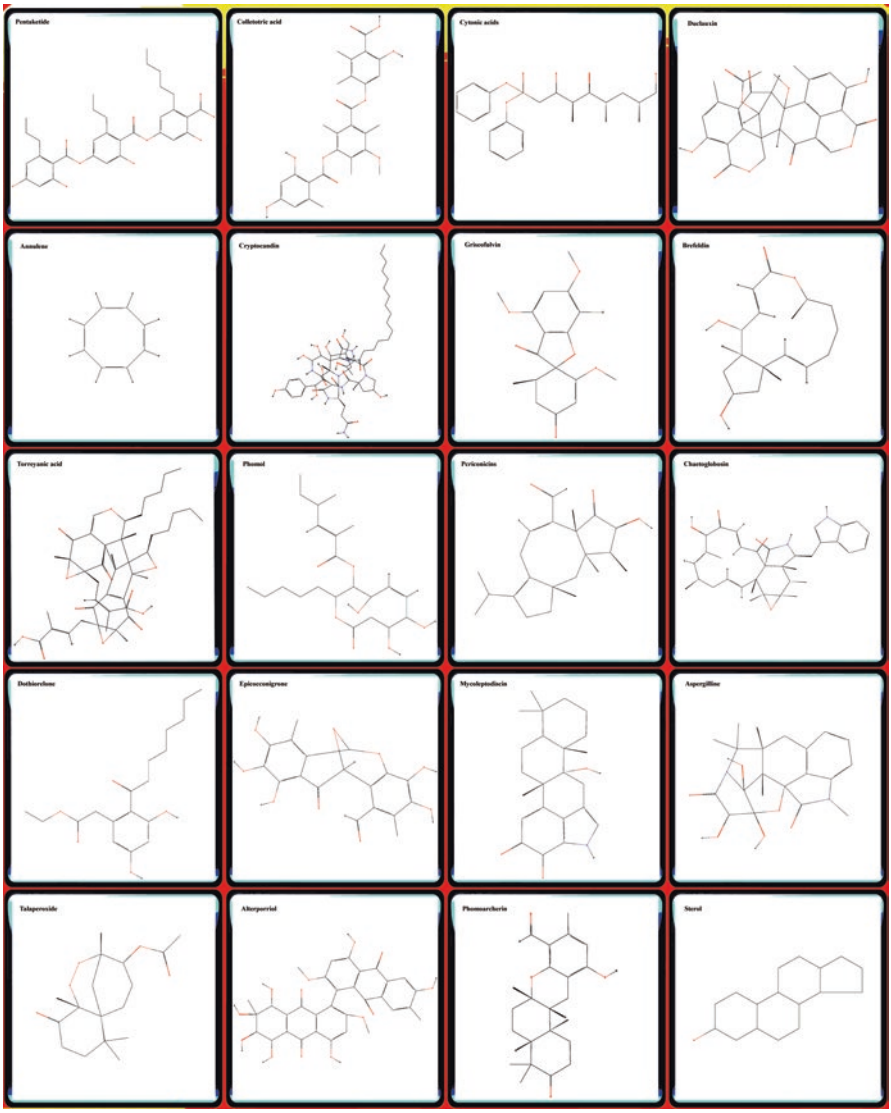

Fig. 7.2 Chemical structures of fungal natural products

7.2 Benefits of Fungi

Fungi cycle nutrients in the environment, and some fungi (e.g., mushrooms) are edible. They also have pharmaceutical and industrial uses (Miles and Chang 2004; Kavanagh 2017).

Fungi feed on dead organic matter including leaf waste, wood, soil, dung, and dead animals. Fungi recover 85% of the carbon from dead organic substance and

free the locked-up nutrients so they can be consumed by other organisms. This function makes fungi important for the continuing health of all environments, defined as a biological ecosystem made up of all the living organisms in a specific area with the nonliving parts with which they interact (Miles and Chang 2004; Kavanagh 2017). A number of mushrooms such as *Agaricus subrufescens*, *Ganoderma lucidum*, and *Cordyceps sinensis* are employed in treatment as therapeutics in traditional Chinese prescriptions. A study published in the *Journal of Natural Products* found that fungi have unique compounds and nutrients that are impressive against viruses. For example, the shiitake fungus is a source of a medical drug named lentinan, which is approved for cancer treatments in Japan. The recognized antibiotic drug penicillin is taken from the fungus *Penicillium*. Pieces of fungus were found near the body of a Neolithic tourist in the Alps; it is theorized that he used a number of fungi as tinder, and other types possibly therapeutically (Kavanagh 2017).

A number of fungi are edible, such as oyster mushrooms, straw mushrooms, shiitakes, truffles, milk mushrooms, and black trumpets. Button and Portobello mushrooms are usually used in soups and salads. Mushrooms enhance the taste of any dish that they accompany. Mushrooms contain a wealth of vitamin D_2, when shown in ultraviolet light. Investigations have indicated that an hour of ultraviolet light radiation just before the mushrooms are harvested increases their vitamin D_2 content (Bilbao-Sainz et al. 2017; Nölle et al. 2017). Fungi are also used to create industrial chemicals such as malic, citric, and lactic acids. They are also used in the manufacture of industrial enzymes such as amylase, cellulase, and lipase; lipase is exploited in laundry detergents. Fungi are also utilized as insect biocontrol agents. Insecticidal toxins manufactured by fungi can kill insects at a very low concentration (Sauer et al. 2008).

7.3 Fungal Enzyme Technology

Another aspect was added to fungal knowledge by the advent of enzyme technology. Buchner and Rapp (1897), a German chemist, indicated that fermentation can happen not only in the existence of yeast cells but also in yeast extracts. This concept was a turning idea in fungal technology because it substantiated the realization that involvement of the entire organism or cell is not absolutely required for a given bioprocess. As more and more living processes (alcoholic fermentation, lactic acid fermentation, respiration, etc.) were shown to be the effects of the progressive action of enzymes, attempts at production, isolation, purification, and commercial utilization of enzymes began to gather momentum. The basics for intentional usage of fungal enzymes had already been laid down by Takamine (1914) with the manufacture of an amylase preparation. A number of fungal enzymes are now manufactured on an industrial scale, including glucose aero-hydrogenase, proteases, pectinases, amylases, lipolases, and cellulases (Kües 2015; Laluce et al. 2018).

Fungal enzymes have established a niche of their own in several industrial processes such as bread making, malting, whey processing, sucrose conversion, starch

conversion, fruit processing, and cheese making. These enzymes are also used as supplements for pancreatic lipase, and for producing soap, lactose-free foods, soft-centred confectionery, and so on. The development of enzyme immobilization techniques helped overcome limitations in the use of enzymes (limited availability, instability, high costs). The first commercial application of immobilized enzyme technology was developed by Tanabe Seiyaku Co. of Japan in 1969 using immobilized L-amino acylase from *Aspergillus oryzae* (Tanaka et al. 1992; Wingard et al. 2014). Gholami-Shabani et al. (2014, 2015, 2016) showed fungal enzymes were capable of producing gold and silver nanoparticles**.**

7.4 History of Fungi Applications in Medicine

A comprehensive account of the study of fungi in folklore and rituals, from prehistoric times to the present day, was given by Wasson (1968). The study of fungi in folklore, fiction, and rituals from prehistoric times to the modern era is called the science of ethnomycology (Singh and Aneja 2012). Throughout the history of mankind, fungi have been regarded with fear and fascination; sometimes revered, sometimes hated, but always considered mysterious. They have been a source of food since times of antiquity, and there are many recipes for cooking fungi in a book written by one Caelius Apicus in the third century A.D. (probably the oldest cookery book written in Europe). However, the artificial cultivation of mushrooms for food does not appear to have been practiced until the seventeenth century. The physician and poet Nicander, born about 150 A.D., wrote (Singh 1999; Macheleidt et al. 2016): The physician Galen expressed his view about fungi as follows: "Fungi after being eaten in large quantities yield cold, clammy, noxious juices as their nourishing qualities; the Boleti are the most harmless and after them the Amanitae, as for the rest it is far safer to have nothing to do with them" (Singh 1999). Moreover, Dioscorides, the celebrated Greek writer on medicine, stated that even the good kinds "if partaken of too freely are injurious being indigestible causing stricture and cholera," and he advised an emetic being taken after meals where they had been eaten (Singh 1999).

The word "fungus" may be derived from "fungus," a corpse, and "ago," I make. The best authenticated and ingenious case of fungal poisoning is that of the emperor Claudius who succeeded Caligula in A.D. 41. Emperor Claudius' fourth wife Agrippina was determined that her son from a former marriage should succeed as emperor instead of the Emperor's son Britannicus. She prepared a dish composed of Amanita of the Caesars steeped in juice extracted from the deadly *Amanita phalloides*. Claudius died of fungal poisoning and Nero succeeded him to the throne (Singh 1999).

The fungus *Fomes officinalis* was thought by Dioscorides (first century A.D.) to be a powerful drug that could relieve almost all complaints. He wrote, "Its properties are styptic and heat-producing, efficacious against colic and sores, fractured limbs and bruises from falls. It is given in liver complaints, asthma, jaundice,

dysentery, kidney diseases and cases of hysteria. In cases of phthisis it is administered in raisin wine, in affections of the spleen with honey and vinegar. By persons troubled with pains in the stomach and by those who suffer from acrid eructations the root is chewed and swallowed without any liquid."

For many centuries fungi were regarded as the result of decomposition, not the cause. However, with the work of C.H. Pearsoon (1775–1835) and E. Fries (1794–1878), a new era in our knowledge of fungi began (Singh 1999). The sudden appearance of so-called fairy rings or the luminosity of certain wood-rotting fungi provided the early herbalists, naturalists, and poets with fascinating material with which to write interesting poems and fiction. The fungus *Fomes fomentarius* has been used as tinder, and its medicinal use in India was introduced by the Portuguese in Goa (Vaidya and Rabba 1993; Singh and Aneja 2012).

The Mexican Indians seem to regard hallucinogenic plants (and mushrooms) as mediators with God, not as a god themselves. However, the Nahuma Aztecs called the mushrooms teonanacatl, meaning 'God's flesh.'

In Vedic times, Soma was drunk by priests only (Wasson 1968; Singh 1999). Some of their hymns are of so exalted, even delirious, a tenor that the modern leader was led to exclaim: "This surely was composed under the influence of a divine inebriant." It takes little perception to sense the difference in tone between awe-inspired hymns to Soma and the rowdy drinking songs of the West prompted by alcohol. "In a word, my belief is that Soma is the divine mushroom of immortality, and that in the early days of our culture, before we made use of reading and writing, when the Rig Veda was being composed, the prestige of this miraculous mushroom ran by word of mouth, far and wide throughout Eurasia, well behind the regions where it grew and was worshipped."

The identity of Soma is *Amanita muscaria* (Fr. ex L.) Quel., in English, fly agaric. The fly agaric has been the sacred element in the Shamanic rites of many tribes of Northern Siberia. Alcohol was introduced by Russians in the sixteenth and seventeenth centuries, but fly agaric had been their precious possession long before then. Mushroom intoxication had a quite different effect from alcoholic drunkenness, as the former put the Kamchatka natives into a peaceful and gentle mood (Singh 1999).

According to Von Maydell (1861–1871) "the fungi produces only a feeling of great comfort, together with outward signs of happiness, satisfaction and well-being. Thus far the use of Fly Agaric has not been found to lead to any harmful results, such as impaired health or reduced mental powers" (Singh 1999).

7.4.1 Primary Fungal Metabolites

Fungi are quite versatile and can use a range of different sources of nutrients, which are assimilated into the primary metabolic pathways at different points. There are a number of commercially important primary metabolites, for example, citric acid, ethanol, enzymes, amino acids, and vitamins. Primary metabolites are formed

during the active growth of the fungus. The fungus will take from the natural environment those nutrients which it can utilize as an energy source to produce materials such as proteins, lipids, and nucleic acids for its continued growth and biomass production (Turner 1971; Walker and White 2017).

7.4.2 Secondary Fungal Metabolites

Some commercially important secondary products, including antibiotics (e.g., penicillin from *Penicillium chrysogenum*, cephalosporin from *Cephalosporium acremonium*, griseofulvin from *Penicillium griseofulvum*) and alkaloids (*Claviceps* spp.), are derived from fungi and used medicinally. Secondary metabolites are not essential for fungal growth but are produced naturally by many fungi. Many of the compounds produced have antifungal and antibacterial activity (e.g., antibiotics, mycotoxins) and may therefore impart a competitive advantage, acting as weapons for survival. The compounds have antimicrobial activity to which producer organisms may well be sensitive. Most fungi have mechanisms to prevent their own demise from the effects of the compounds they produce. In most cases the products are formed after active growth, and by that time the mycelium is able to detoxify the compound or prevent entry of the antibiotic through the cell wall by a change in the permeability of the plasma membrane (Macheleidt et al. 2016).

7.5 Fungal Discovery for Pharmaceutical Purposes

Mushrooms are an important group of nutraceuticals used for a great variety of purposes (Rathore et al. 2017). Besides their edibility, fungi have long been considered to have medicinal properties. Having a unique composition, fungi have had an important part in folk medicines as therapy for a variety of ailments. As part of the diet, they are excellent for sufferers of diabetes, obesity, hyperacidity, hypertension, atherosclerosis, high blood pressure, anaemia, and constipation. A large number of mushroom species including *Ganoderma lucidum*, *Coriolus versicolor*, *Fomes fometarius*, *Tremella fociformis*, and *Lentinus edodes* are traditionally used in Chinese folk medicine. Other mushrooms, including *Agrocybe cylindracea*, *Tricholoma mongolicum*, *Inonotus obliquus*, *Pleurotus ostreatus*, *Collybia dryophila*, *Collybia radicata*, *Collybia peronata*, *Suillus bovinus*, *Coprinus plicatilis*, *Hypholoma fasciculare*, *Leucopaxillus giganteus*, and *Pholiotina appendiculata*, are being explored extensively for their pharmaceutical utility. Scientists are now paying considerable attention to investigation of the medicinal utility of plants in general but to mushrooms in particular. A large number of bioactive substances from fungi which are effective against microbes (fungi, bacteria, viruses) have already been identified.

7.6 Bioactive Natural Products of Fungal Origin

There is considerable interest in obtaining new products from natural ecosystems. In fungi, besides enzymes of biotechnological utility and other products including biocontrol agents, the metabolites of pharmaceutical utility are of great interest for counteracting common ailments. Table 7.1 summarizes Fungi and their natural products possessing biological activity. Besides antibiotics, a large number of substances known as host defense potentiators (HDPs), protein-bound polysaccharide, or polysaccharide–protein complexes (PSPCs) have been isolated from mushrooms (Subramanian 1995). Such bioactive mushroom metabolites are believed to be able to aid in the revitalization of our immune system against a large number of pathogenic and nonpathogenic diseases. These metabolites are reported to act as biological response modifiers with the capability to activate macrophages and T cells, and to produce cytokines, interleukins, and tumour necrosis factors. Some such reported bioactive substances from mushrooms are pleurotin, lepiochlorin, clavicin, sparassol, triterpenes, ganoderols, armillarin, dictyophorin, cylindan, adenosine, etc. Applications of various bioactive metabolites derived from fungi are discussed next.

7.7 Application of Fungal Metabolites as Antimicrobials

Recently, among the microorganisms, fungi have been accepted as one of the best resources for new active bioactive compounds that are important defenses against a number of pathogenic bacteria and fungi (Deshmukh et al. 2017). Penicillin was the first and most important discovery, which proved to have an effective action against gram-positive bacteria (Hautbergue et al. 2018). The crude extract of *Aspergillus ochraceus* and *Penicillium citrinum* displayed extensive spectral antibacterial properties, inhibiting developing germs, specifically *Pseudomonas aeruginosa*. Hypericin ($C_{30}H_{16}O_8$), a naphthodianthrone-derived compound, and emodin ($C_{15}H_{10}O_5$), thought to be the main pioneer for the synthesis of hypericin, in a fungus isolated from a pharmaceutical plant had antimicrobial activity against a number of bacteria and fungi, including *Staphylococcus* sp., *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Salmonella enterica*, *Escherichia coli*, and the fungal organisms *Aspergillus niger* and *Candida albicans* (Ratnaweera et al. 2015; Malhadas et al. 2017).

Nascimento et al. (2015) reported 156 fungal isolates distributed across 19 taxa obtained from 468 fragments of *Calotropis procera* leaves at different stages of maturation. The rate of fungus colonization increased with leaf age and development. The dominant fungal species of *C. procera* reported in Northeast Brazil were different from those found in studies on similar species and other species of the similar genus in native areas. The main fungus was *Phaeoramularia calotropidis* (63%), followed by *Guignardia bidwellii* (21%). Seven isolates of fungi displayed

Table 7.1 General features of bioactive fungi and fungal natural products

Fungi and applications	Products	References
Antimicrobial activity		
Fusarium sp.	CR377, a pentaketide	Brady and Clardy (2000)
Colletotrichum gloeosporioides	Colletotric acid	Chapla et al. (2014)
Colletotrichum gloeosporioides	Secondary metabolites	Zou et al. (2000)
Cytonaema sp.	Cytonic acids A and B human cytomegalovirus protease inhibitors	Guo et al. (2000)
Gliocladium sp.	Annulene	Stinson et al. (2003)
Streptomyces munumbi	Munumbicins A, B, C, D	Castillo et al. (2002)
Cryptosporiopsis cf. *quercina*	Cryptocandin	Strobel et al. (1999)
Xylaria sp. F0010	Griseofulvin	Park et al. (2005)
Streptomyces sp. NRRL 30566	Kakadumycins	Castillo et al. (2003)
Cladosporium sp.	Brefeldin A	Wang et al. (2007)
Streptomyces sp.	Coronamycin	Ezra et al. (2004)
Pestalotiopsis microspora	Torreyanic acid	Strobel et al. (2002)
Phomopsis sp.	Phomol	Huang et al. (2008)
Periconia ericonia	Pcriconicins A and B	Kim et al. (2004)
Anticancer activity		
Chaetomium globosum L18	Chaetoglobosin X	Wang et al. (2012)
Dothiorella sp.	Dothiorelone	Du and Su (2014)
Epicoccum nigrum	Epicocconigrone A	El Amrani et al. (2013)
Periconia sp. F-31	Periconiasin A, B	Zhang et al. (2013)
Diaporthe sp.	Diaporine A	Song et al. (2014)
Penicillium manginii YIM PH30375	Duclauxamide A_1	Cao et al. (2015)
Penicillium chrysogenum QEN-24S	Penicitide A	Gao et al. (2010)
Acremonium camptosporum	Acremoxanthone E	Meléndez-González et al. (2015)
Chaetomium globosum TY1	Chaetomugilide A, B, C	Li et al. (2013)
Mycoleptodiscus sp.	Mycoleptodiscin B	Ortega et al. (2013)
Eurotium rubrum	12-Demethyl-12-oxo-eurotechinulin B	Yan et al. (2012)
Aspergillus versicolor	Aspergilline A, B, C	Zhou et al. (2014)
Phomopsis glabrae	PM181110	Verekar et al. (2014)
Pestalotiopsis foedan	(4*S*, 8*S*, 4*R*, 8*R*)-Foedanolide	Yang and Li (2013)
Myrothecium roridum	Myrotheciumone A	Lin et al. (2014)

(continued)

Table 7.1 (continued)

Fungi and applications	Products	References
Phomopsis sp. (ZH76)	3-*O*-(6-*O*-α-L-Arabinopyranosyl)-β-D-glucopyranosyl-1,4 dimethoxyxanthone	Huang et al. (2013)
Talaromyces flavus	Talaperoxide B, D	Li et al. (2011)
Alternaria sp.	Alterporriol K, L	Huang et al. (2011)
Phomopsis archeri	Phomoarcherin B, C	Hemtasin et al. (2011)
Penicillium brocae MA-231	Penicibrocazine A, B, E, F	Meng et al. (2014)
Fusarium sp.	5-Hydroxyl dihydrofusarubin A, B	Kornsakulkarn et al. (2011)
Anti-diabetic activity		
Agaricus bisporus	Dehydrated fruiting body extracts	Jeong et al. (2010)
Agaricus campestris	Aqueous fruiting body extract	Gray and Flatt (1998)
Agaricus subrufescens (*A. blazeimurril*, *A. brasiliensis*)	β-Glucans and enzymatically produced oligosaccharides	Niwa et al. (2011)
Agaricus sylvaticus	Aqueous fruiting body extract	Costa-Fortes and Carvalho-Garbi-Novaes (2011)
Cerrena unicolor	Extracellular polysaccharide	Yamac et al. (2009)
Coprinus comatus	4,5-Dihydroxy-2-methoxybenzaldehyde (comatin)	Ding et al. (2010)
Cordyceps militaris	Polysaccharide-enriched fraction of fruiting body	Zhang et al. (2006)
Cordyceps takaomantana (Paecilomyces tenuipes)	Fruiting body extract containing 4-β-acetoxyscirpendiol (ASD)	Yoo and Lee (2006)
Ganoderm alucidum sensu lato	(3β, 24*E*)-Lanosta-7,9(11),24-trien-3,26-diol (ganoderol B)	Fatmawati et al. (2011)
	Water extracts of polysaccharides from fruiting bodies	Jia et al. (2009)
	Water extract of whole fruit body	Seto et al. (2009)
Grifola frondosa	Mushroom extracts rich in vanadium	Cui et al. (2009)
	Glycoprotein extract (SX-fraction)	Preuss et al. (2007)
Hericium erinaceus	Methanol extract of the mushroom	Wang et al. (2005)
Inonotus obliquus	Culture broth	Sun et al. (2008)
	Ethyl acetate fraction	Xu et al. (2011)
	Terpenoid and sterol compounds	Lu et al. (2010)
Laetiporus sulphureus var. *miniatus*	Crude extracellular polysaccharides (EPS)	Hwang and Yun (2010)
Phellinus merrillii	Ethanol extracts	Huang et al. (2011)
Sparassis crispa	Freeze-dried fruiting body samples	Yamamoto and Kimura (2010)

antimicrobial activity against human and plant pathogens. The antibacterial action was indicated to be stronger than the antifungal activity.

Meng et al. (2017) isolated fungi from fresh leaves of *Dioscorea nipponica* Makino to determine their antimicrobial activity. Antimicrobial activity from the

isolated fungus was detected using the filter paper method against *Staphylococcus aureus, Staphylococcus albus, Staphylococcus citreus, Micrococcus tetragenus, Escherichia coli, Salmonella typhi, Pseudomonas aeruginosa, Bacterium termo, Bacillus endocarditis capsulatus, Shigella flexneri*, and *Bacterium paratyphosum* B. The antibacterial activity of these fungi isolated from leaves of *Dioscorea nipponica* Makino could be exploited for the development of new antibacterial biological agents.

The fungi provide a wide diversity of antifungal metabolic compounds that have important actions against a number of pathogenic fungi. Altomare et al. (2000) isolated two alpha pyrones, antifungal compounds named fusapyrone and deoxyfusapyrone, from *Fusarium semitectum* with strong antifungal activity against a number of pathogenic or mycotoxogenic filamentous fungi such as *Alternaria alternata*, *Aspergillus flavus*, *Botrytis cinerea*, *Cladosporium cucumerinum*, *Phoma tracheiphila*, and *Penicillium verrucosum*. *Streptomyces* sp. produces the bioactive compound polyenes which have broad-spectrum activity against *Aspergillus* sp., *Candida* sp., etc. (Hay 2003; Vicente et al. 2003). Amphotericin B, nystatin, and natamycin are the main polyenes used extensively for the cure of diseases such as coccidioidal meningitis, cutaneous dermatophytes, and histoplasmosis and in the treatment of mycotic disease (Gupte et al. 2002; Iznaga et al. 2004; Gohel et al. 2006). Recently, Wu et al. (2015) isolated the two new antifungal and cytotoxic components (4*S*,6*S*)-6-[(1*S*,2*R*)-1,2-dihydroxybutyl]-4-hydroxy-4-methoxytetrahydro-2*H*-pyran-2-one, (6*S*,2*E*)-6-hydroxy-3-methoxy-5-oxodec-2-enoic acid, and three other compounds, LL-P880, LL-P880, and ergosta-5,7,22-trien-3b-ol, from the secondary metabolites of *Dendrobium officinale*. The results of the investigation indicated compounds one through four display prominent antifungal properties against the tested microbes *Cryptococcus neoformans*, *Candida albicans*, *Aspergillus fumigatus*, and *Trichophyton rubrum*. Huang et al. (2001) screened fungi having antifungal activity that were isolated from the inner bark of three pharmaceutical plants, *Taxus mairei*, *Cephalotaxus fortunei*, and *Torreya grandis*, collected from Fujian Province, China. Antifungal activity was determined by observing fungal growth inhibition: 52.3% of fungi fermentation broths displayed growth inhibition on at least one pathogenic fungus, such as *Neurospora* sp., *Trichoderma* sp., or *Fusarium* sp. Among all fungi isolated, the genus *Paecilomyces* has the highest positive rate of antifungal activity.

Liu et al. (2010) isolated 262 strains of fungi from 23 evergreen plant species collected from Zijin Mountain in Nanjing, China. Of the fungi isolates, 203 were classified into 23 taxa in 19 genera on the basis of colony morphology and microscopic observation of mycelia and asexual/sexual spores. The greatest richness was obtained from *Cedrus deodara*, whereas the highest diversity of identified species was isolated from *Sabina procumbens*. Some fungi appeared to be host specific, such as *Botrytis ricini* lt300, *Geotrichum candidum* lt274, and *Lacellina graminicola* lt256, although other strains (e.g., *Alternaria alternata* lt222, *Anthina* sp. Lt147, *Colletotrichum gloeosporioides* lt305, *Fusarium solani* lt293) were commonly isolated from a range of plants. The richness of the fungi recovered from plant branches was significantly higher than those from leaves. Moreover, about 70% of the

obtained fungi could produce antifungal metabolites against at least one plant pathogenic fungus. The EtOAc extracts of the seven species *Anthina* sp. lt147, *Colletotrichum gloeosporioides* lt305, *Ectostroma* sp. Lt144, *Fusarium decemcellulare* lt183, *Fusarium oxysporum* lt173, *Paraconiothyrium brasiliense* lt161, and *Colletotrichum montemartini* lt198 showed broad inhibition against the growth of all six phytopathogens, with inhibition rates from 20% to 80%.

Bai et al. (2017) isolated 16 fungal strains from *Erigeron canadensis*, one of the traditional Chinese medicines used to treat pathogenic infection and dysentery, which were evaluated for their antifungal activities against one human pathogen, *Candida albicans*, and two phytopathogens, *Colletotrichum fructicola* and *Rhizoctonia cerealis*. The bioassay effects showed that the ethyl acetate extract of the fermentation broth of these fungi had stronger antimicrobial activity. Among these fungi strains, the ethyl acetate extracts of two strains, NPR003 and NPR005, displayed the strongest inhibitory special effects and has potential application in the detection of novel antifungal agents. Yu et al. (2018) isolated 81 fungi strains from different parts (leaves, bark, fruits) of *Camellia oleifera* from Hunan Province (China) to define their species conformation and potential as organic and biological control agents of *C. oleifera* anthracnose.

The fungi were recognized by morphological and phylogenetic examination. Fungal colonization rates of the leaves, barks, and fruit were 58%, 27%, and 14%, respectively. The isolates were recognized as 14 genera, belonging to two parts, *Deuteromycotina* and *Ascomycotina*; 88% of all isolates belonged to *Deuteromycotina*. The dominant fungal species, occurring with a high relative frequency, were *Pestalotiopsis* sp. (14.81%), *Penicillium* sp. (14.81%), and *Fusarium* sp. (12.35%). The Simpson's and Shannon's variety indices exposed the highest species variety in the leaves, followed by the barks and fruits. The resemblance index for the leaves against barks comparison was the highest, signifying that the number of fungal species collected from leaves and bark was higher than that from fruits. Based on the effects of dual culture investigations, only five strains displayed antifungal activity against *C. oleifera* anthracnose disease, with isolate ty-64 (*Oidium* sp.) producing the biggest inhibition zones. These results indicate that fungi could be a promising source for antifungal bioactive agents. Figure 7.3 shows the mechanisms of antimicrobial action by fungal natural compounds.

7.8 Application of Fungi as Antiviral Agents

In contrast to bacterial infectious diseases, viral diseases cannot be treated by common antibiotics and specific drugs are urgently needed. Antiviral effects are described not only for whole extracts of mushrooms but also for isolated compounds. These effects could be caused directly by inhibition of viral enzymes, synthesis of viral nucleic acids, or adsorption and uptake of viruses into mammalian cells. These direct antiviral effects are exhibited especially by smaller molecules. Indirect antiviral effects are the result of the immunostimulating activity of

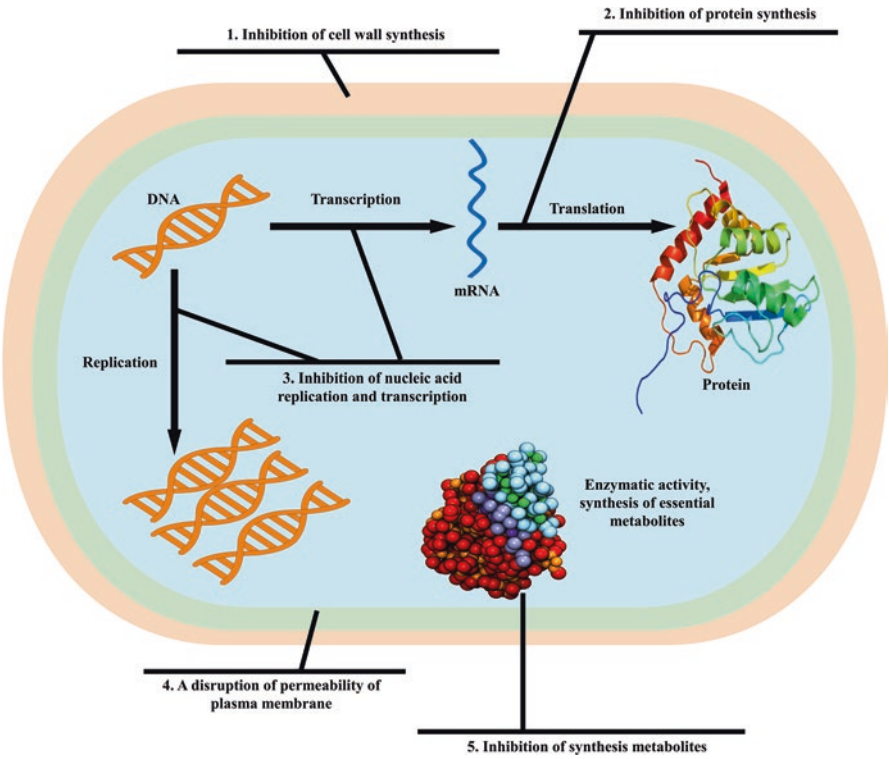

Fig. 7.3 Mechanisms of antimicrobial action of fungal natural products

polysaccharides or other complex molecules (Brandt and Piraino 2000; Piraino 2006). In vitro antiviral activity against influenza viruses types A and B was demonstrated for mycelial extracts of *Kuehneromyces mutabilis* (Mentel et al. 1994), extracts and two isolated phenolic compounds from *Inonotus hispidus* (Awadh Ali et al. 2003), and ergosterol peroxide, present in several mushrooms (Lindequist et al. 2005). The antiviral activity of *Collybia maculata* (vesicular stomatitis viruses in BHK cells) is caused by purine derivatives (Leonhardt et al. 1987).

Of the large variety of mushrooms tested against the poliomyelitis virus in mice, some of them, namely, *Boletus frostii*, *Calvatia gigantea*, *Chlorophyllum molybdites*, *Lepiota morgani*, *Russula emetica*, *Panaeolus subbalteatus*, *Armillaria mellea*, *Coprinus micaceus*, *Agaricus campestris*, and *Agaricus placomyces* have been reported to possess significant potential. In *Calvatia gigantea*, some high molecular weight derivatives are reported to be effective against poliomyelitis and influenza viruses (Miles and Chang 2004). Hobbs (2000) reported the interferon-inducing capability in a *Lentinus edodes* extract. In this fungus, eritadenine has been reported to be active against the influenza virus in mice. Kahlos (1996) reported that the black thin external surface of *Inonotus obliquus* strains (AlHINI, AlH3N2′ AlEquine 2, BNamagata/16/18) grown in birch showed 100% inhibition against human

influenza viruses A and B and horse influenza virus A. The antiviral activity of this fungus is thought to be caused by betulin, lupeol, and mycosterols.

7.9 Application of Fungi as Anti-HIV Properties

Most antiretroviral drugs currently in use to treat an HIV infection are chemically synthesized and lead to the development of viral resistance, as well as cause severe toxicities. However, a largely unexplored source for HIV drug discovery is fungi that live in a symbiotic relationship with plants. These fungi produce biologically active secondary metabolites, which are natural products that are beneficial to the host (Wellensiek et al. 2013). The extract of *Grifola frondosa* has been shown to kill the AIDS virus and is reported to be capable of enhancing the activity of helper-T cells. The extract of this fungus is reported to be as effective against HIV as the widely used toxic drug azidothymidine (AZT) (Nanba et al. 2000). Lentinan from *Lentinus edodes* also possesses the ability to enhance host resistance to a variety of infections including HIV-l (Subramanian 1995). Walder et al. (1995) reported the strong anti-HIV-1 activity of aqueous extracts from *Fomitella supina*, *Phellinus rhabarbarinus*, *Trichaptum perrottetii*, and *Trametes cubensis*. The active principle is reported to have acted by the mechanism of direct virion inactivation and by inhibition of syncytium formation. The unknown active components of these extracts individually or in combination may have therapeutic relevance. Collins and Ng (1997) isolated a polysaccharopeptide (PSP) from *Coriolus versicolor* that has potential for use against HIV-1 infection. It acts by inhibition of the interaction between HIV-l group 120 and the immobilised CD4 receptor (IC_{50} = 150 μg/ml), recombinant HIV-I reverse transcriptase (IC_{50} = 125 μg/ml), and glycohydrolase enzyme associated with viral glycosylation. Such properties, coupled with its high solubility in water, heat stability, and low cytotoxicity, make it a useful compound for controlling HIV infections.

Wellensiek et al. (2013) reported several hundred extracts from fungi of desert plants and evaluated the inhibitory effects on HIV-1 replication of those extracts that showed less than 30% cytotoxicity in T lymphocytes. Those extracts that inhibited viral replication were fractionated to isolate the compounds responsible for activity. Multiple rounds of fractionation and antiviral evaluation lead to the identification of four compounds, which almost completely impede HIV-1 replication. These studies demonstrate that metabolites from fungi of desert plants can serve as a viable source for identifying potent inhibitors of HIV-1 replication.

Zhao et al. (2014) reported a novel laccase was isolated and purified from fermentation mycelia of mushroom *Coprinus comatus* with an isolation procedure including three ion-exchange chromatography steps on DEAE-cellulose, CM-cellulose, and Q-Sepharose, and one gel filtration step by fast protein liquid chromatography on Superdex 75. The purified enzyme was a monomeric protein with a molecular weight of 64 kDa that possessed a unique N-terminal amino acid sequence of AIGPVADLKV, which has considerable high sequence similarity with

that of other fungal laccases but is different from that of the *C. comatus* laccases reported. The enzyme manifested an optimal pH value of 2.0 and an optimal temperature of 60 °C using 2,2′-azinobis(3-ethylbenzothiazolone-6-sulfonic acid) diammonium salt (ABTS) as the substrate. The laccase displayed, at pH 2.0 and 37 °C, K_m values of 1.59 mM towards ABTS. It potently suppressed proliferation of tumor cell lines HepG2 and MCF7 and inhibited human immunodeficiency virus type 1 (HIV-1) reverse transcriptase (RT) with an IC_{50} value of 3.46 μM, 4.95 μM, and 5.85 μM, respectively, signifying that it is an antipathogenic protein.

Pang et al. (2017) reported that 33 metabolites including 5 phenalenone derivatives (1–5), 7 cytochalasins (6–12), 13 butenolides (13–25), and 8 phenyl derivatives (26–33) were isolated from *Aspergillus* sp. CPCC 400735 cultured on rice. The plant diseases caused by fungi include rusts, leaf rot, and stem rots, which may cause severe damage to important crops of all compounds, as elucidated by nuclear magnetic resonance (NMR), mass spectroscopy (MS), and circular dichroism (CD) experiments, of which 1–5 (asperphenalenones A–E), 6 (aspochalasin R), and 13 (aspulvinone R) were identified as new compounds. Specifically, asperphenalenones A–E (1–5) represent an unusual structure composed of a linear diterpene derivative linked to a phenalenone derivative via a C–C bond. Compounds 1, 4, 10, and 26 exhibited anti-HIV activity with IC_{50} values of 4.5, 2.4, 9.2, and 6.6 μM, respectively (lamivudine 0.1 μM; efavirenz, 0.4×10^{-3} μM).

7.10 Application of Fungi as Hypocholestrolemic and Hypolipidemic Agents

Some of the edible mushrooms, for example, *Lentinus edodes*, *Agaricus bisporus*, *Pleurotus florida*, *P. ostreatus*, and *Auricularia auricula*, are reported to possess the ability to lower blood cholesterol. Suzuki and Oshima (1976) reported the hypocholestrolemic effects of shiitake in humans. Bhandari et al. (1991) recommended *Pleurotus florida* as the potential source of active ingredients required for sufferers of high blood cholesterol. The hypolipidemic properties of shiitake are reported to result from eritadenine (= lentysine, lentinacin), 2(*R*),3(*R*)-dihydroxy-4-(9-adenyl)-butyric acid (Okumura et al. 1974). In *Auricularia polytricha*, an anti-platelet substance (adenosine) has been reported to inhibit platelet aggregation (Markheja and Bailey 1981). Inclusion of dried *Agaricus bisporus* sporophores at a level of 5% or 10% in the diet of rats has been reported to have resulted in the accumulation of lipids in the liver with simultaneous decrease in the circulatory lipids, except phospholipids, in plasma. Bobek et al. (1995) reported the antioxidative effect of the oyster mushroom in hypercholesterolemic rats. Alam et al. (2011) reported that the *Pleurotus salmoneostramineus* diet supplement provided health benefits by acting on the atherogenic lipid profile in hypercholesterolemic rats. Yoon et al. (2012), reported that feeding a diet containing a 5% powder of the fruiting bodies of *P. salmoneostramineus* in hypercholesterolemic rats reduced plasma total cholesterol,

triglyceride, low-density lipoprotein, total lipid, phospholipids, and LDL/HDL ratio by 22.55%, 51.38%, 69.23%, 29.67%, 16.61%, and 65.31%, respectively. The mushroom also significantly reduced body weight in hypercholesterolemic rats. Moreover, it had no adverse effects on plasma albumin, total bilirubin, direct bilirubin, creatinine, blood urea nitrogen, uric acid, glucose, total protein, calcium, sodium, potassium, chloride, inorganic phosphate, magnesium, and enzyme profiles. Feeding the mushroom increased total lipid and cholesterol excretion in feces. The plasma lipoprotein fraction, separated by agarose gel electrophoresis, indicated that *P. salmoneostramineus* significantly reduced plasma β and pre-β-lipoprotein, whereas it increased α-lipoprotein. A histological study of liver tissues by conventional hematoxylin and eosin and oil red O staining showed normal tissue in mushroom-fed hypercholesterolemic rats. This study suggests that the *P. salmoneostramineus* diet supplement provided health benefits by acting on the atherogenic lipid profile in the rats.

7.11 Application of Fungi as Anti-Diabetic Agents

Diabetes is a chronic disease causing severe health problems to millions worldwide and has become a significant ailment in many countries (Wild et al. 2004; WHO 2011; Hagopian et al. 2011; Smith et al. 2012). According to the WHO (2011), diabetes mellitus accounts for 2.2% of deaths in the world and is one of the main causes of death among humans. The most recent data released by the Center for Disease Control and Prevention (CDC) reports that diabetes is the seventh leading cause of death in the United States; diabetes affects 25.8 million (8.3%) of the US population (CDC 2011). Medicinal fungi have been valued as a traditional source of natural bioactive metabolites over many centuries and have been targeted as potential anti-diabetic and hypoglycemic anti-diabetic agents. Bioactive metabolites including polysaccharides, proteins, dietary fibers, and many other biomolecules isolated from medicinal mushrooms and their cultured mycelia have been shown to be successful in diabetes treatment as biological anti-hyperglycemic agents. The polysaccharide (β-glucans) contained in fungi, in particular, can restore the functions of pancreatic tissues, causing an rise in insulin output via the functional β cells, thus lowering the blood glucose levels, and it has also been shown to improve the sensitivity of peripheral cells to circulating insulin (Misra et al. 2009; Qiang et al. 2009; Xiao et al. 2011). Health-conscious diets can incorporate mushrooms as ideal low-energy foods for diabetes patients as they contain very low amounts of, or are lacking, fats and cholesterol, are low in carbohydrates, and high in proteins, vitamins, and minerals (Mattila et al. 2002; Guillamón et al. 2010; Phillips et al. 2011a, b; Ulziijargal and Mau 2011; Smiderle et al. 2012). Mushrooms are also known to contain certain compounds that aid in the proper functioning of the liver (Wani et al. 2010), pancreas, and other endocrinal glands, thereby promoting the regulation of insulin and associated hormones to ensure healthy metabolic functioning (Wasser and Weiss 1999; Smiderle et al. 2012). Most medicinal fungi such as *Agaricus*

subrufescens, *A. bisporus*, *Cordyceps sinensis*, *Coprinus comatus*, *Ganoderma lucidum*, *Inonotus obliquus, Coprinus comatus*, *Phellinus linteus*, *Poria cocos, Pleurotus* spp., and *Sparassis crispa* have been reported to have hypoglycemic effects on reducing blood glucose levels and anti-diabetic effects (Cha et al. 2006; Yang et al. 2008; Seto et al. 2009; Jeong et al. 2010; Kim et al. 2001a, b; Lu et al. 2010; Yamamoto and Kimura 2010; Lee et al. 2010; Li et al. 2011a, b).

Edible mushrooms are known for their low calorific value (25–30 calories/1.00 g fresh weight) and low carbohydrate content in comparison to other food items (Saini and Atri 1999). For this reason they are considered excellent for diabetic patients. A freeze-dried powder containing mycelia of *Ganoderma lucidum* has been shown to lower blood sugar levels in experimental diabetic rats. Three hypoglycemic principles, namely, ganoderans A, B, and C, are reported to have been isolated from the fruit bodies of *G. lucidum*, and these have been characterized as peptidoglycans. Of these ganoderan-B is considered to be the most important insofar as anti-diabetic properties are concerned (Subramanian 1995).

7.12 Role of Fungi in Blood Building and Immunity

For centuries, fungi have been used as food and medicine in different cultures. More recently, various bioactive compounds have been isolated from diverse types of fungi. Among these, immunomodulators have attracted much attention based on the growing development of the immunotherapy sector. Fungi immunomodulators are categorized under four groups based on their chemical nature: lectins, terpenoids, polysaccharides, and proteins. These compounds are produced naturally via fungi cultivated in greenhouses. For effective industrial production, cultivation is carried out in submerged culture to increase bioactive compound yield, decrease production time, and reduce the cost of downstream processing (Lee et al. 2012; El Enshasy and Hatti-Kaul 2013).

Mushrooms contain vitamins of the B-complex (Crisan and Sands 1978). Folic acid, which is a blood-building vitamin, is good for persons suffering from anaemia. Ascorbic acid (vitamin C), present in edible mushrooms, increases resistance in the human body (Crisan and Sands 1978). Along with these, other vitamins (for example, pantothenic acid and niacin) and minerals (calcium, phosphorus, potassium, copper, iron, etc.) add to the vitality and immunity of the body (Chang and Miles 1989). Rowan et al. (2003) reported a protein-bound polysaccharide (PSP) from *Coriolus versicolor* with immunopotentiating effects when administered at 2 g/kg/day to rats. The active principle is reported to have restored the cyclophosphamide-induced immunosuppression such as depressed lymphocyte proliferation.

Agaricus blazei Murill (AbM) is an edible, medicinal mushroom of Brazilian origin. It is used traditionally against a range of diseases, including cancer and chronic hepatitis, and has been cultivated commercially for the health food market. AbM has recently been shown to have strong immunomodulating properties, which has led to increasing scientific interest (Hetland et al. 2008).

7.13 Application of Fungi as Hepatoprotective Agents

Extracts of *Ganoderma lucidum* have been shown to be hepatoprotective. Apart from liver regeneration, beneficial effects in counteracting hepatic necrosis and hepatitis have been reported. Ganodosterone from *G. lucidum* is reported to be a liver protectant with the ability to stimulate liver function. Similarly, ganoderic acids T, S, and R from *G. lucidum* and triterpenoids from *G. tsugae* (lucidone-A, lucidenol, ganoderic-B, ganoderic acid C2) are reported to be hepatoprotective (Soares et al. 2013; Sharma and Annepu 2018). Wasser (2002) reported that polysaccharides from the mycelium of *G. lucidum* are promising agents for the inhibition of hepatic cirrhosis. They further suggested that these polysaccharides could be promising anti-fibrotic agents because of their capability to lower the collagen content, serum aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP), and total bilirubin in the liver.

7.14 Application of Fungi as Anticoagulant and Antithrombic Agents

An antiplatelet substance (adenosine) from *Auricularia polytricha* is known to inhibit platelet aggregation (atherosclerosis) and prolong bleeding time. The ingestion of this fungus as food is reported to reduce the chances of heart attack (Jacob et al. 1980). *Auricularia* is said to have been used in folk medicine in Hong Kong to thin the blood and reduce clotting problems in postpartum women (James et al. 1987). The oral administration of a fructo-oligosaccharide mixture from *Lentinus edodes* (SK-204) to rats for 10 weeks is reported to have antithrombic action (Otsuka et al. 1996) by the promotion of fibrinolysis and thrombolysis. Román et al. (2017) reported a fucogalactan from *Agaricus bisporus* that was sulfated by two methodologies based on an optimized sulfation method. The direct action of chlorosulfonic acid and SO_3-pyridine complex during the sulfation reaction and its effects on anticoagulant activity were evaluated. Chemical sulfations produced two sulfated fucogalactans, E100 and ESL, respectively. Clotting assays (APTT, PT, TT) showed that both sulfated polysaccharides have anticoagulant activity, and that ESL was more potent compared to E100. The FXa (factor Xa), T, and FXIIa (factor XIIa) activities in the presence of the sulfated polysaccharides were determined. The better anticoagulant activity of ESL could be related to anti-FXIIa activity and also probably to its higher bioavailability. The HPSEC analysis showed similar MW of 1.08×104 g mol^{-1} and 1.00×104 g mol^{-1} for E100 and ESL, respectively. NMR and methylation analyses indicated a heterogeneous sulfation pattern for E100, whereas ESL showed conserved unsulfated (1 → 6)-linked α-D-Galp residues in the main chain and a more homogeneous sulfation pattern. The DS values of ESL and E100 were 1.0 and 2.8, respectively, indicating that the sulfation pattern is more important for the anticoagulant activity than the amount of sulfate.

7.15 Application of Fungi as Insecticidal Agents

Fungal entomopathogens are important biological control agents worldwide (Vega et al. 2012). Natural infections by fungi have a major role in the control of many economic insect pests. Occasionally, the resultant disease reaches epizootic levels, causing a complete collapse of the pest population. Figure 7.4 illustrates schematic mechanisms of action of fungal metabolites against insects. Biological pesticides are often touted as being safer and more sustainable than their chemical counterparts. Specific species of fungi can function as parasites of insects. Fungal pathogens naturally attack many insect species, and in some respects they are well suited to development as biopesticides. When a fungus is used as an insecticide, it is named a "mycoinsecticide" (Ortiz-Urquiza et al. 2015). Fungi can be mass produced in vitro, then stored for long periods, and their spores applied with conventional spray equipment. In contrast to viruses and bacteria, which must be ingested to infect insects, they infect simply through external contact. Also, compared with

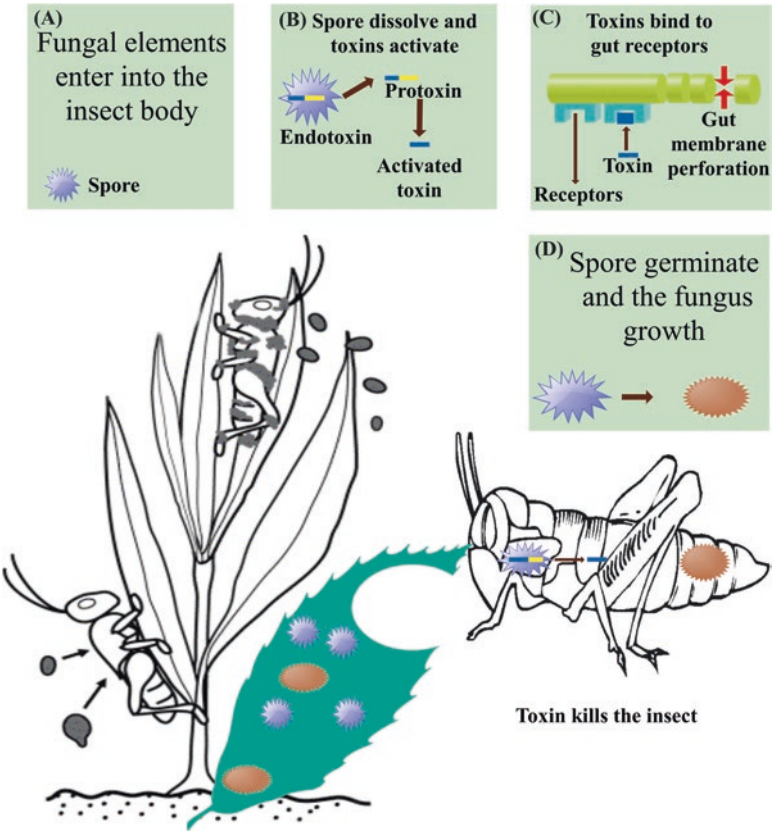

Fig. 7.4 Mode of insecticidal action of fungal natural products

most chemical insecticides, fungi are less toxic to mammals and have negligible environmental impacts (Thomas and Read 2007).

Fungal-based biopesticides act on a higher level. Numerous biopesticides contain parasitic fungi, the kind that grow inside an insect body and feed on its internal tissue until it dies (sometimes beyond that), which is particularly helpful when compared with artificial pesticides, which often contain toxic chemicals such as chlorine, arsenic, formaldehyde, and ammonia. Fungi, on the other hand, are alive, and they could evolve along with the insects that they are being used to control, which means pesticide resistance may become less of an issue. Some synthetic pesticides have been shown to have harmful effects on the environment and human health. One family of pesticides, the neonicotinoids, is being blamed for the decline in bee populations during the past decade. In recent years, crop protection has been trending towards integrated pest management (IPM) by microorganisms, fungi, and bacteria as insecticides. Approximately 1000 species of fungi are pathogenic to insects (Purwar and Sachan 2006) but only a limited number have been utilized for use as insecticides. Some species of fungi used as insecticides are *Beauveria* spp., *Metarhizium* spp., *Trichoderma*, *Isaria* spp., *Lecanicillium* spp., and *Purpureocillium* spp. *Beauveria bassiana* effectively targets the pecan weevil, Colorado potato beetle, and kudzu bug, among other pests. The fungus *Metarhizium* (the green muscardine fungus) is often used in the field, protecting crops from beetle grubs, wireworm, corn root worms, and countless other insects. One variant is currently being used to progress biopesticides including a line using a mycopesticide that can cause a mushroom to develop from the dead body of a pestto distribute spores that warn other insects. The fungi then burrow into the bugs using their hyphae. The hyphae spread the insectotoxins throughout the insect to activate them, eventually leading to the insect's death (Vega 2018).

7.16 Other Applications of Fungal Natural Products

In addition to the foregoing applications, fungi are finding an increasing role in various other areas in therapeutics and investigation. Coatney et al. (1953) described terpenoids of *Clitocybe illudens* to be effective against *Plasmodium gallinaceum*. Researchers reported insecticidal properties of an amino acid derivative, tricholomic acid, from *Tricholoma muscarium*. Aqueous extracts of *Pleurotus sajor-caju* have been reported to reduce the rates of nephron deterioration in persons suffering from renal failure (Saini and Atri 2012). *Grifola frondosa* has been reported to be beneficial for lowering blood pressure, diabetes, and constipation. Another fungus, *Fomes officinalis*, has been listed as a universal remedy for a variety of ailments. The spores and capillitia of *Lycoperdon* are known for use in stopping bleeding from wounds (Saini and Atri 2012). Singh et al. (2014) isolated a hypotensive and vasorelaxing lectin from *Tricholoma mongolicum*.

This lectin, on administration to rats at a dose of 10 mg/kg body weight, reduced arterial blood pressure. The hypotensive activity of lectins is reported to be mediated

through vasorelaxation via adenosine A_2 receptors or nitric oxide production. *Inonotus hispidus*, which produces styrylpyrones (hispidin) and derivatives of caffeic acid (hispolon) as pigments, has been suggested as a valuable source of new drugs (Lee and Yun 2011). Two novel eudesmane-type sesquiterpenes, dictyophorines A and B and a known compound teucrenone isolated from *Dictyophora indusiata*, have been reported to promote nerve growth fraction (Saini and Atri 1999). Eleven species of bracket mushrooms belonging to the genus *Phellinus* (*P. badius*, *P. chinchonensis*, *P. durrissimus*, *P. gilvus*, *P. linteus*, *P. merrilli*, *P. pachyphloes*, *P. pectinatus*, *P. robiniae*, *P. senex*, *P. sublinteus*) and two species of *Ganoderma* (*G. applanatum* and *G. lucidum*) are reported to be in extensive use as *Phanasomba* or *Phanas alombe* by the *Ayurvedic Vedas*. A paste prepared from these is applied to the gums to stop excessive salivation and has been reported to act as a good styptic (Vaidya and Lamrood 2000).

7.17 Conclusion and Future Prospects

Economically natural products and their associated compounds account for an important portion of worldwide business. Recent progress in genetics and molecular biology and applications to bio-combinatorial artificial/natural products will produce unprecedented novel natural products. Different isolation and detection technologies will accelerate the discovery of drugs from fungal metabolites. With the help of these novel methods, natural products will be even more important as a source variety for the pharmaceutical and agricultural industries. Fungi are worthy and reliable sources of new natural compounds with a high level of biodiversity and can also produce numerous compounds with pharmaceutical impact, which is currently attracting scientific research worldwide. Secondary metabolites produced by fungi can be applied as substitutes and sustainable sources of these compounds. However, the commercial implication of manufacture of desirable metabolite compounds by fungi remains a future goal. A deeper understanding of fungi at the molecular and genetic levels, of biogenetic gene cluster regulation, and of the effects of environmental changes and culture conditions on gene expression, will be useful for optimizing secondary metabolite manufacturing by fungi under laboratory conditions. Further research at an advanced molecular level may suggestion better insights into fungal biodiversity and the regulation of fungal secondary metabolism.

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Chapter 8
Fungal-Derived Natural Product: Synthesis, Function, and Applications

Amit Kumar Singh, Harvesh Kumar Rana, and Abhay K. Pandey

8.1 Introduction

Fungi are a group of eukaryotic organisms that neither belong to plant nor animal kingdom and obtain their nutrition by absorbing and decaying organic matter. Kingdom *Fungi* includes highly diverse organism, of which very less has been described at scientific level (Bass and Richards 2011). They are present in almost every ecological niche, making them the second largest kingdom after bacteria. It has been reported that earth is approximately estimated to have 1.5 million species of fungi and only 10% of it is known to scientific community (Hibbett et al. 2007; Stajich et al. 2010). Structural diversity of fungus ranges from unicellular yeast to multicellular higher fungi like molds and mushroom. A characteristic feature of all the fungi is the presence of chitin in their cell wall; like animals they are also heterotrophs, cannot synthesize their own food, and acquire their food by absorption through hyphae by secreting digestive enzymes (Blackwell 2011). Since the discovery and evaluation of penicillin, an antibiotic of fungal origin, researchers around the globe have been searching for fungal-derived natural bioactive products having nutritional and pharmaceutical properties. Fungi are vast and yet untapped sources for pharmaceutically important product having activities such as anticancerous, antioxidant, hepatoprotective, antibacterial, antidiabetic, etc. In addition to their pharmaceutical properties, higher fungi such as mushroom are being analyzed for their nutritional attributes like low fat and high protein, fiber, and vitamin content (Cheung 2010). Fungal classification provides important information related to their evolution and systematics. Fungal kingdom is classified into *Chytridiomycota*, *Zygomycota*, *Ascomycota*, and *Basidiomycota*. Figure 8.1 shows phylogenetic relationships of major groups in the kingdom *Fungi*.

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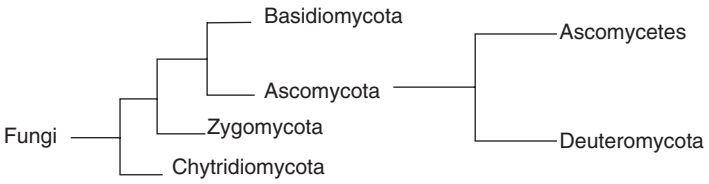


Fig. 8.1 Phylogenetic relationships of major groups in the kingdom *Fungi*

Ascomycota, also known as sac fungi, a phylum of kingdom *Fungi* contains a saclike structure, the ascus, which contains four to eight ascospores in the sexual stage. Ascomycetes reproduce through formation of conidia, a non-motile structure. *Ascomycota* is the largest phylum of kingdom *Fungi* and reported to have more than 60% of the total described species: some of them are pathogenic to the plant, animal, and humans, for example, *Candida albicans*, *Nematospora*, and *Cryptococcus neoformans* (Ma et al. 2013; Gauthier and Keller 2013); some industrially important fungi, for example, *Aspergillus*, *Fusarium*, and *Trichoderma* (Punt et al. 2002; Dufossé et al. 2014; Van Den Berg et al. 2010 and Archer 2000); and several others industrially important secondary metabolites producing strains (Rana et al. 2018a, b; Yadav et al. 2017; Evidente et al. 2014; Bräse et al. 2009; Bladt et al. 2013). *Basidiomycota* commonly known as club fungi are the most advanced and characterized phylum including mushroom-forming fungi. They include around 30% of the reported fungal species. Sexual reproduction unlike ascomycetes does not involve sexual organ; instead plasmogamy occurs. Basidiomycetes are the best wood decomposers, and they also show symbiotic relationship with plants (e.g., mycorrhizal fungi) (Morel et al. 2013). *Chytridiomycota*, earlier known as chytrids, are the division of zoosporic organism in kingdom *Fungi*. Their reproduction takes place by means of zoospores. Chytrids are saprobic and sometime act as parasites. Zygomycetes are mostly terrestrial in habitat and absorb nutrients from soil, decaying plant, or animal material. About 1050 zygomycetes are reported, and they reproduce by means of zygospore formation (Plett and Martin 2011).

8.2 Fungi as Sources of Natural Products

Fungi produce a variety of natural products, including all important categories of natural products, i.e., terpenes, alkaloids, sesquiterpenoids, and sugars (Table 8.1), and among them antimicrobial natural products are of particular interest due to reduced effectiveness of available antibiotics toward bacterial infection, a serious threat to worldwide health security (Aiken et al. 2014). Several fungal secondary metabolites are of human welfare; the β-lactam antibiotics are one of the most widely used antibiotics in the world (Suman et al. 2016; Yadav et al. 2018; Hoffmeister and Keller 2007; Hamad 2010). Despite the fact that very few fungal species have been examined for their secondary metabolite production, many

Table 8.1 Some important examples of secondary metabolites produced by fungi (Jiang and Zhiqiang 2000)

Source organism	Secondary metabolite	Other industrial products
Penicillium sp.	Penicillin	Penicillin, the most widely used antimicrobials agent
Cephalosporium acremonium	Cephalosporin C	Cephalosporin, antibacterial agents
Aspergillus sp.	Mevinolin	Lovastatin, simivastatin, Lipitor, and other HMG-CoA enzyme inhibitors
Tolypocladium niveum	Cyclosporine	Cyclosporine A, immunosuppressant
Penicillium sp.	Mycophenolic acid	Immunosuppressant
Penicilllium griseofulvum	Griseofulvin	Antifungal agent
Strobilurus tenacellus	Strobilurins	Agricultural fungicides
Fusidium coccineum	Fusidic acid	Antibacterial agent
Claviceps purpurea	Ergot alkaloids	Ergotamine used for migraine and Ergonovine for obstetric treatment
Fusarium graminearum	Zearalenone	Growth promoter in cattle
Canddida galbrata	Echinocandin	Antifungal drug, inhibits the synthesis of glucan in the cell wall via noncompetitive inhibition of the enzyme 1,3-β glucan synthase
Fusarium moniliform	Gibberellins	Gibberellins, plant growth hormone

remarkable lead structures have been identified for the production of pharmaceutically and agriculturally important agent of fungal origin. Studies reported that approximately 40 drugs available in Australian domestic market are of fungal origin. However, among these 40 drugs, many are penicillin derivatives, which revolutionized the treatment of microbial diseases (Beekman and Barrow 2014). In addition to pharmaceutical utility, fungi are very useful in producing crop protecting agents such as strobilurin origin fungicides, which constitute a large part of crop protecting agents sold worldwide (Henningsen 2003). Taking into consideration that very few fungal species have been explored for potential secondary metabolite production, numerous fungal species are untapped sources of natural products with potential bioactivity for the development of pharmaceutical and crop protecting agents.

Some fungal secondary metabolites are having cholesterol lowering property like statin. They act either through selective inhibition of enzyme squalene synthase or by hampering the activity of HMG-CoA reductase, an enzyme that catalyzes the rate-limiting step of cholesterol biosynthesis (Istvan and Deisenhofer 2001), for example, meavstatin produced from *Penicillium citrinum* and lovastatin of *Aspergillus terreus* origin (Manzoni and Rollini 2002). Cyclosporin, a non-ribosomal

peptide, isolated from *Tolypocladium niveum* has immunomodulatory property and is widely being used in transplant surgery to avoid organ failure (Weber et al. 1994). Fingolimod, a derivative of myriocin, shows activity against multiple sclerosis (Cohen et al. 2010). In some cases, fungal secondary metabolites are not used in their natural form but are subjected to derivatization to produce synthetic derivatives having enormous diversity. Alternatively, these metabolites may also serve as a model for the development of completely synthetic derivatives, for example, strobilurin A, polyketide isolated from the *Strobilurus tenacellu*s (Anke et al. 1977), which inspired the discovery and exploitation of β-methoxy-acrylic acid, forming the basis of the strobilurin fungicides (Fig. 8.2). This class of antifungals includes one of the world's most sold fungicides, azoxystrobin (Bartlett et al. 2002).

8.3 Synthesis of Fungal Natural Products

The kingdom *Fungi* produces a vast variety of secondary metabolites including all important classes: terpenes, alkaloids, polyketides, and sugars. Some of the reported synthesis strategies are discussed in the following.

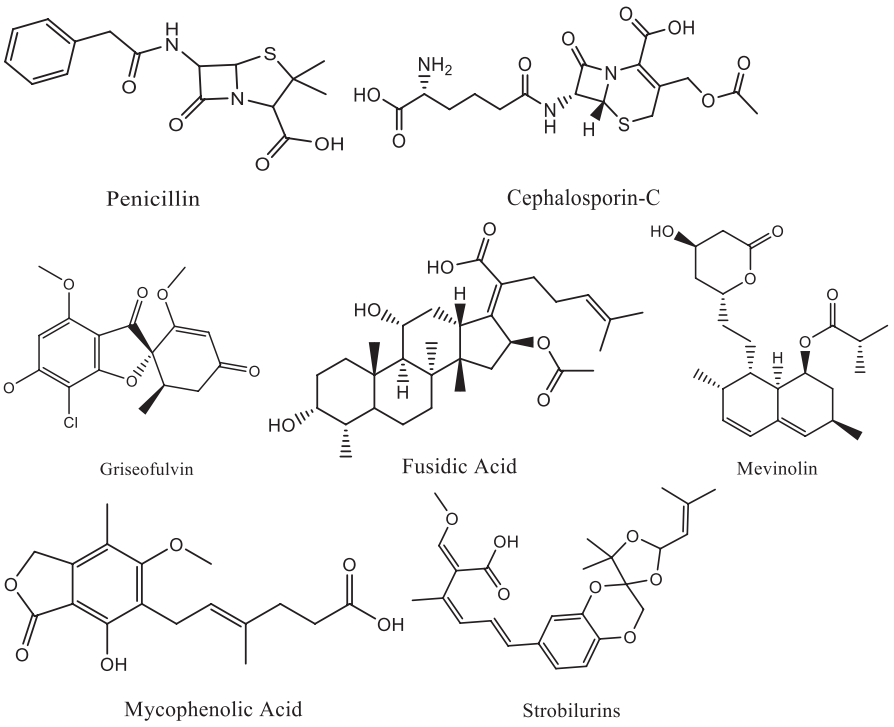


Fig. 8.2 Structure of some fungal-derived secondary metabolite

8.3.1 Terpenes

The fungal communities are still a largely unexplored territory for discovering newer secondary metabolites and their biosynthetic pathways, together with isoprenoid-derived secondary metabolites. Particularly this is true for basidiomycetes; they are very difficult to grow under laboratory conditions, and excluding some species they are not amenable to genetic manipulation. Yet with these drawbacks, mushrooms are being used in traditional medicine since ages and reported to have a variety of bioactive metabolites having antimicrobial, cytotoxic, and anticancer compound (Singh et al. 2018; Zjawiony 2004). The chemical class of terpenes is prevalent in fungi. Numerous compounds of class terpenes have been isolated and identified and are of great scientific interests because of their biochemical properties.

All terpenes and terpenoids of fungal origin are derived from five-carbon intermediate isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP), synthesized from acetyl coenzyme A (CoA) through mevalonate pathway. Further it is followed by a head to tail condensation of IPP units to DMAPP to give isoprenyl diphosphates (10C-geranyl pyrophosphate, 15C-farnesyl pyrophosphate, or 20C-geranylgeranyl pyrophosphate); this reaction is catalyzed by isoprenyldiphosphate synthases. However, longer chains (C30, C40) are synthesized by head to tail condensation of two farnesyl pyrophosphate or two geranyl-geranyl pyrophosphate, and the reaction is catalyzed by squalene synthase and phytoene synthase, respectively (Lindequist et al. 2005; Xu et al. 2010). These linear compounds are substrates of many different enzymes that either transfer the prenyl residue to another molecule, mostly aromatic compounds, or initiate the cyclization of prenyl chain giving rise to tens of thousands of different isoprenoid derived secondary metabolites. There are several classes of prenyl transferases and cyclases in fungi related to production of different natural products. Paxiline indolediterpene biosynthetic pathway requires two types of prenyl transferases for prenylation reaction (Zhong and Xiao 2009; Alves et al. 2012). Terpenoids of different classes can be distinguished on the basis of scaffolds, either derived solely from IPP units or from mixed origin. Farmer group is divided into mono-, di-, sesqui-, or triterpenoids containing 2–6 IPP units; this group is also characterized by the presence of carotenoids and some rare sesquiterpenoids, and the latter group includes prenylated aromatic secondary metabolites (Abraham 2001; Elisashvili 2012; Wasser 2011).

8.3.2 Sesquiterpenoids

Sesquiterpenoids are 15-carbon-length structurally diverse natural compounds isolated from plant, fungi, and bacteria. They are synthesized from farnesyl pyrophosphate catalyzed by enzyme sesquiterpene synthase (Christianson 2008). The enzyme sesquiterpene synthases has a conserved active site structure, aspartate rich motifs

coordinated by Mg^{2+} cluster and the enzyme bind to the pyrophosphate group of farnesyl via Mg^{2+} cluster and after binding to the pyrophosphate group the orientation of prenyl chain changes towards hydrophobic cavity of the enzyme. This orientation results in conformational changes and causes active site closure followed by concomitant cleavage of pyrophosphate to give rise to transoid allylic carbocation (Davis and Croteau 2000; Cane and Kang 2000; Christianson 2006 and Vedula et al. 2008). This carbocation gets transferred to isoprenyl chain and is ultimately quenched by proton abstraction or by water molecule. Folding of isoprenyl chain is determined by binding pocket of enzyme, thereby defining the product profile of a particular sesquiterpene synthase. Enzymes catalyze different initial cyclization reactions to produce cyclic carbocation intermediates, for example, trans-humulyl-carbocation, which is a 1, 11-cyclization product. Secondary carbocation produced can undergo additional cyclization and rearrangement until quenching in the active site occurs (Lesburg et al. 1998).

Filamentous fungi, for example, *Aspergillus*, *Penicillium*, and *Fusarium* sp., produce mycotoxins which impose severe health risks on humans and animals. Several well-characterized mycotoxins are sesquiterpenoids like chemical structures and play an important role in determining fungal virulence. PR toxin, phomenone, and PR toxins are produced by *Aspergillus* and *Penicillium* strains. Several studies have been done to elucidate the biosynthetic mechanism of mycotoxins (Cane and Kang 2000). However, the detailed knowledge about the biosynthetic pathway of mycotoxins were elucidated in blue cheese mold *P. roqueforti* by screening a genomic phage library and found that a gene cluster is involved in encoding PR toxin (Hidalgo et al. 2014). Table 8.2 lists the common sesquiterpenoids of fungal origin and their synthesis strategies.

8.3.3 Diterpenoids

Diterpenoids are of 20C chain length and generated from cyclization of geranylgeranyl diphosphate by either one- or two-step separate cyclase activities. Diterpenoids which are cyclized by one-step cyclase require class I diterpene synthase which catalyzes ionization-dependent diphosphate cleavage followed by carbocation displacement and quenching like sesquiterpene synthases. Conserved aspartate-rich motifs in these enzymes likewise facilitate Mg-ion mediated binding of the diphosphate group. However, in two-step diterpenoids synthesis two separate enzymes are involved. First, a class II-type protonation driven reaction mechanism to give rise carbocation at the terminal C14-C15 double bond of the prenyl diphosphate chain that is cyclized into a bicyclic diphosphate characteristic of labdane-related Diterpenoids (Peters 2010). Second, a class I ionization-driven cleavage of the diphosphate group is followed by carbocation-triggered cyclization to yield the final cyclic scaffold. Fungi have a large bifunctional enzyme having both class I and class II activities. Class I (α-domain) terpenoid synthases present at the N-terminal region, while C-terminal region has α-barrel (or γβ) domains of class II terpenoid

Table 8.2 Common fungal-derived sesquiterpenoids, their source organisms, and synthesis strategies

Chemical compounds	Source	Synthesis strategies	References
Alliacol A	*Marasmius alliaceus*	In 2004, Mihelcic and Moeller accomplished the first enantioselective total synthesis by utilizing an anodic coupling reaction, proving its usefulness in synthesis, in establishing the required stereochemistry, as well as an electrochemical cyclization, followed by Friedel–Crafts alkylation, to generate the tricyclic framework	Mihelcic and Moeller (2004)
Pasteurestins A and B	*Agrocybe cylindracea*	A chiral building block precursor or a Reformatsky-type reaction was used to generate the required enantiomerically pure building blocks, with a subsequent [2 + 2 + 2] cycloaddition forming the tricyclic skeleton	Kogl et al. (2008)
Cheimonophyllon E	*Cheimonophyllum candidissimum*	They applied aldol chemistry and dihydroxylation as key steps to generate cheimonophyllon E in 13 steps with 3.5% overall yield	Kogl et al. (2008)
Tremulenolide A	*Phellinus tremulae*	Ashfeld and Martin applied a rhodium-catalyzed cyclopropanation, allylation, and [5 + 2]-cycloaddition cascade to produce Tremulenediol A in 16 steps with 6% overall yield	Ashfeld and Martin (2005)
Lagopodin A	*Coprinus lagopus*	In order to find a way to generate the unusual core structure, Johnson–Claisen rearrangement reaction has been performed to obtain lagopodin A in 12 steps with 26% overall yield	Srikrishna et al. (2006)
Isovelleral	*Lactarius vellereus*	Gained scientific interest because of its biochemical properties and its unusual bicyclic framework. Magnesium iodide-induced rearrangement—cyclopropanation cascade—was utilised to produce the framework; isovelleral was obtained in 12 steps with 10% yield, starting from a known precursor	Magnusson et al. (1972); Bell et al. (2001)
Hypnophilin	*Pleurotellus hypnophilus*	It possesses interesting antitumor activity, hypnophilin was chosen by Paquette and Geng as a target to apply their newly developed squarate ester cascade reaction in 2002	Paquette and Geng 2002
Cryptoporic acid A	*Cryptoporus volvatus*	In order to study its antitumor activity, first total synthesis of cryptoporic acid A methyl ester was done by Hashimoto and colleague	Hashimoto et al. 1987 and Tori et al. 2000

synthases. Actually, characteristic fungal diterpene synthases are not fused, and cyclization process involves class II (γβ) and class I (α) terpenoid synthases for this two-step cyclization mechanism (Smanski et al. 2012). Table 8.3 denotes some common fungal-derived diterpenoids and their synthesis mechanism.

8.3.4 Triterpenoids

Triterpenoids include a large group of chemical compounds and are predominantly present in animal and plant kingdom. Essential role of triterpenoids is to function as membrane sterols in eukaryotes to provide membrane fluidity. Animal, plant, and fungal kingdom are reported to have three kinds of sterols: phytosterols (sitosterol, stigmasterol, campestrol) in plants, cholesterol in animals, and ergosterol in fungi (Dupont et al. 2012; Yadav 2018). Majority of fungal-derived triterpenoids' natural products have been isolated from basidiomycetes; however, certain ascomycetes are

Table 8.3 Common fungal-derived diterpenoids, their source organisms, and applications

Chemical compounds	Source organism	Application	References
Fusicoccin A	*Phomopsis amygdali*	Binds and permanently activates plasma membrane H + -ATPase, which causes severe physiological effects in plants. Binds to a highly conserved family of 14-3-3 proteins in eukaryotes, which regulate a wide range of cellular functions	de Boer and de Vries-van Leeuwen (2012)
Cotylenin A	*Cladosporium spp*	Binds and permanently activates plasma membrane H + -ATPase, which causes severe physiological effects in plants. Interaction with 14-3-3 proteins has been shown to induce differentiation of leukemia cells and apoptosis of cancer cells	de Boer and de Vries-van Leeuwen (2012)
Sarcodonin G	*Sarcodon scabrosus*	Exhibits the greatest inhibition of HeLa cell viability and cell proliferation, with anti-inflammatory activity along with anti-proliferative effects on human cancer cells	Mei et al. (2009)
Erinacine B-E	*Hericium erinaceus*	Antibacterial activity, cytotoxic effect on cancer cells and compounds that stimulate the synthesis of the nerve growth factor (NGF), anti-carcinogenic, and as well reducing the metabolism of fats	Kawagishi et al. (1994)
Chaxine A	*Agrocybe chaxingu*	Playing a central role in the formation of the skeleton and regulation of its mass. Bone forming cells, or osteoblasts, have an equally important role in the regulation of bone mass	Kawagishi et al. (2006)
Clavilactone B	*Clitocybe clavipes*	Acts as a kinase inhibitor against Ret/ptc1 and epidermal growth factor receptor (EGF-R) tyrosine kinases	Arnone et al. (1994)

reported having triterpenoids, for example, fusidane, an antibiotic isolated from *Microsporum canis* (Zhao et al. 2013; Rios et al. 2012). Species such as *Ganoderma*, *Innonotus*, *Daedalea*, *Wolfiporia extensa*, *Laetiporus sulphureus*, and *Antrodia* are famous sources of diverse lanosterane like triterpenoids, having pharmacological properties including antitumor, apoptotic, and antimalarials (Rios et al. 2012). *Ganoderma lucidum*, the medicinal mushroom, is the source of one of the well-known triterpenoid, i.e., ganoderic acid (Boh et al. 2007), and substantial effort has been done to produce gandoric acid via fermentative production processes (Xu et al. 2010). *Aspergillus fumigatus* member of ascomycota produces fusidane-type triterpenoids having antibacterial property, as well as helvolic acid having antibacterial and anticancer activities derived from the protostadienol macrocyclic scaffold (Fig. 8.3). Both the research groups Mitsuguchi et al. and Lodeiro et al. in the year 2009 identified the corresponding triterpene synthase as well as fusidane biosynthetic gene cluster. Cyclization product of triterpene synthase can be willingly changed by single amino acid mutation, and it can be changed either toward lanosterol or protostadienol (Kimura et al. 2010). *Hypholoma sublaterium* commonly called as brick cap are inedible and poisonous and reported to produce clavaric acid. Both the enzymes squalene epoxidase and oxidosqualene synthase are involved in clavaric acid synthesis (Godio and Martin 2009; Godio et al. 2007).

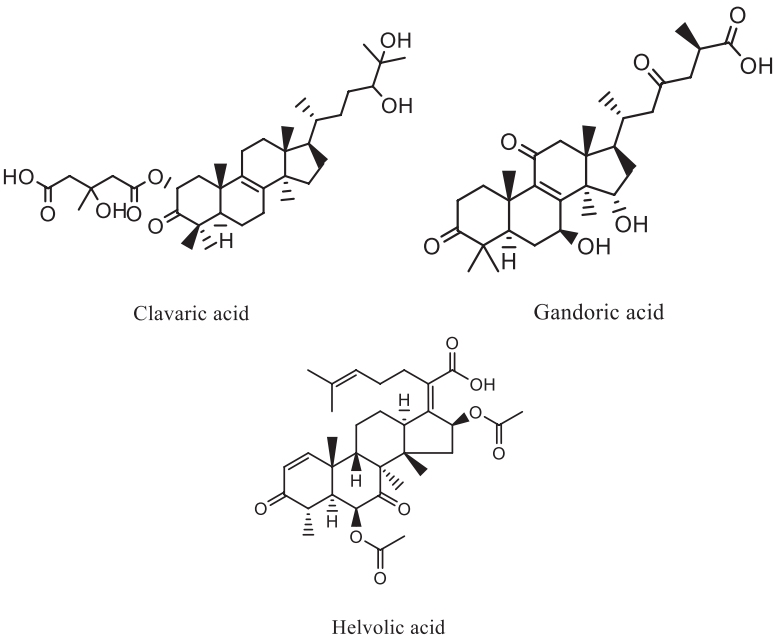


Fig. 8.3 Structure of triterpenoids

8.3.5 Alkaloids

Alkaloids are nitrogenous cyclic organic and are physiologically active as poisons and drugs. Plants are the chief source of alkaloids; however, some are originated from bacteria, fungi, and animals. Alkaloids are secondary metabolites, and they are not involved in growth and reproduction of organism; however, their important function is acting as protective agents against insects and herbivores because of their bitterness and toxicity, so they can be utilized as bio-insecticides and bio-pesticides. In case of nitrogen deficiency, it can be used as a source of nitrogen. Amino acids such as tryptophan, tyrosine, phenylalanine, lysine, ornithine, and anthranilic acid are the precursors of most alkaloids. There are several ways of alkaloid biosynthesis, and it's difficult to classify them. However, there are a few archetypal reactions involved in the biosynthesis of various classes of alkaloids, including synthesis of Schiff bases and Mannich reaction.

Claviceps purpurea is an ergot fungus and grows in the ears of rye. Consumption of contaminated grains or seeds with ergot sclerotium leads to a disease condition called ergotism in humans and other mammals. It mostly infects outcrossing species such as rye as well as triticale, wheat, and barley. However, oats are rarely affected. Ergotamine, ergometrine, ergonaline, and other clavine alkaloids are produced by this group of fungi. Ergotamine, has vasoconstrictors like activity (Fig. 8.4). Ergotamine was first isolated by Arthur Stoll in 1918, and the biosynthesis of ergotamine in fungi requires amino acid L-tryptophan and dimethylallyl diphosphate. The initial step of ergot alkaloid biosynthesis is prenylation of L-tryptophan, catalyzed by enzyme tryptophan dimethylallyl transferase. Now the enzymes methyltransferase and oxygenase catalyze the formation of ergoline and lysergic acid. Lysergyl peptide synthetase covalently links the lysergic acid to amino acids phenylalanine, proline, and alanine. After this spontaneous or enzyme-catalyzed cyclization, oxidation, and isomerization at particular residue lead to formation of ergotamine (Tfelt-Hansen et al. 2000).

Ergotamine causes peripheral vasoconstriction and damages the peripheral epithelium. At higher concentration, ergotamine is advantageous for treating vascular stasis, thrombosis, and gangrene. Ergotamine causes uterine constriction and sometimes is used therapeutically immediately post-partum to decrease uterine bleeding. It is also prescribed for treating migraines. Cafergot, a combination of caffeine and ergotamine, is the common prescription. However, ergot alkaloids at higher doses or consumption of infected grains lead to ergotism, and symptoms include spasms, diarrhea, vomiting, headaches, etc.

Psilocybin is a naturally occurring psychedelic prodrug produced by many species of mushrooms, collectively known as psilocybin mushrooms (Fig. 8.4). The most potent are members of the genus *Psilocybe*, such as *P. azurescens*, *P. semilanceata*, and *P. cyanescens*, but psilocybin has also been reported to be present in several other groups of fungi. Psilocybin at high dose is toxic and the general symptoms include euphoria, hallucinations, changes in perception, a distorted sense of time, spiritual experiences, nausea, and panic attacks. The intensity and duration of psilocybin toxicity are variable and depend upon mushroom cultivar or species,

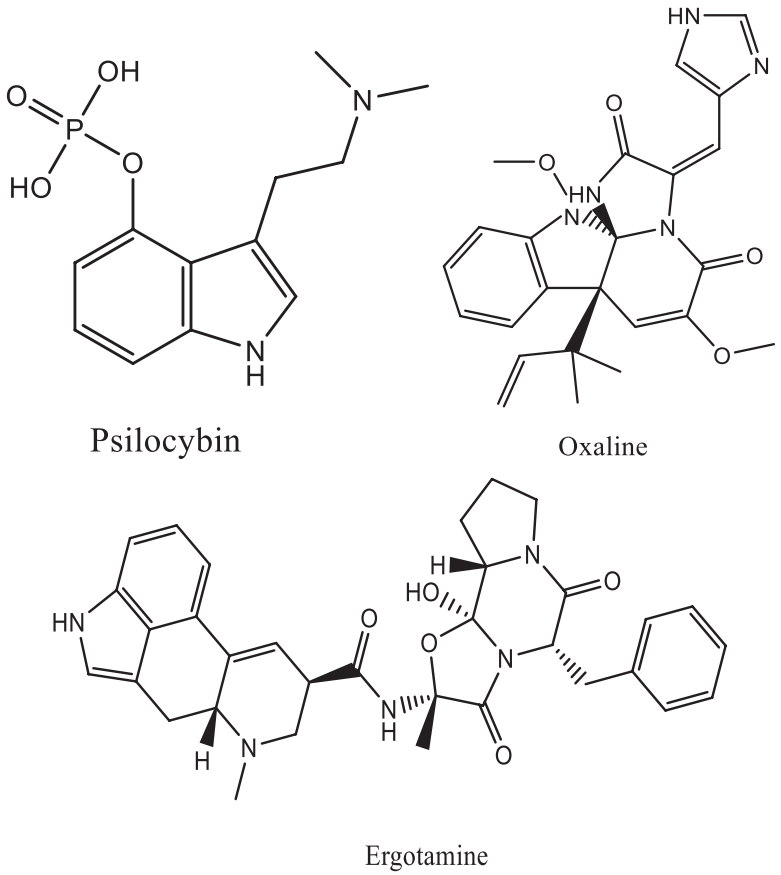


Fig. 8.4 Structure of fungal-derived alkaloids

dosage, and individual immunity (Santos dos et al. 2016). When consumed, psilocybin is quickly metabolized to psilocin, which then binds to the brain serotonin receptors. The hallucinating effect of psilocybin usually lasts from 2 to 6 hours, although it may seem to influenced individuals that the effect last as it since the drug can alters the perception of time. Psilocybin has low toxicity and low harm potential. Possession of psilocybin-containing mushrooms has been outlawed in most countries, and it has been classified as a scheduled drug by many national drug laws.

Meleagrin and glandicolins are known structural analogs. They show the presence of a methoxy group, unusual coupling between tryptophan and dehydrohistidine, as well as a carbon atom bounded by three different nitrogen atoms and a reversed isoprenyl moiety. They are produced by *Penicillium* species (Nozawa and Nakajima 1979; Kawai et al. 1984; Kozlovsky et al. 1994). *Penicillium oxalicum* and *Aspergillus japonicus* are the producers of alkaloid oxaline and neoxaline, respectively (Steyn 1970; Hirano et al. 1979). Structural features of these alkaloids

include presence of a methoxy group, coupling between tryptophan and dehydrohistidine, as well as a carbon atom bounded by three different nitrogen atoms and a reversed isoprenyl moiety like meleagrin and glandicolins (Nagel et al. 1974, 1976; Konda et al. 1980). These alkaloids show structural dissimilarity to well-known G2/M arrest inducers such as colchicine, vinblastine, and taxol (Correia 1991 and Iwasaki 1993). Therefore, its structural uniqueness encouraged scientists around the world to study its biological activity and mechanism of action involved. Oxaline treatment results in cell cycle arrest at M phase of cell cycle, as it inhibits the microtubule/tubulin polymerization. Oxaline is a fungal-derived anticancer compound. It is a derivative of meleagrin, both being benzylisoquinoline alkaloids. It is isolated from deep sea *Penicillium* species. Diketopiperazine made up of tryptophan, dehydrohistidine, and roquefortine is the precursor of biosynthesis of oxaline (Scott et al. 1976; Mantle et al. 1983; Steyn and Vleggaar 2004). Diketopiperazines such as tryprostatin A and (−)-phenylahistine inhibit microtubule polymerization and ultimately cell cycle arrest. Tryprostatine is composed of tryptophan and proline, while (−)-phenylahistine is made up of phenylalanine and dehydrohistidine. Both these compounds have similar structural features; however, their mechanism of action varies. Tryprostatin A inhibits the interaction between tubulin and microtubule-associated protein family tau (MAP 2/tau), and (−)-phenylahistine mechanism of action is similar to colchicine (Usui et al. 1998; Kondoh et al. 1998).

8.3.6 Sugar Derivatives

Sugar derivatives are widely reported in plant species, but some studies also show their presence in kingdom *Fungi*. Cyclophellitol, a polyhydroxy epoxide, was first reported in culture filtrates of *Phellinus* species in 1990 (Atsumi et al. 1990). Recently it attracted serious attention because of its potent β-glucosidase enzyme inhibitory activity. Precursor of cyclophellitol is D-xylose, and Hansen and colleague in 2005 synthesized cyclophellitol from D-xylose in nine steps as shown in Fig. 8.5. Cyclophellitol is a carbocyclic analogue of D-glucopyranose with an epoxide ring on the β-face of the molecule. The inhibition of β-glucosidase is irreversible, which is presumably due to protonation and ring opening of the epoxide by a carboxylate in the active site of the enzyme (Hansen et al. 2005).

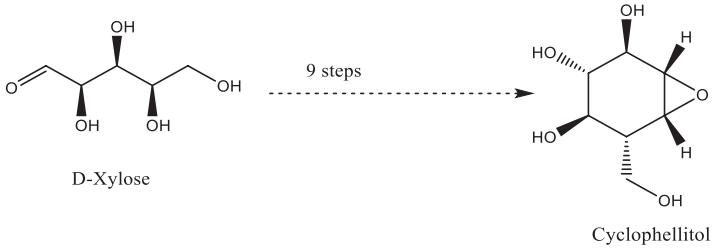


Fig. 8.5 Synthesis of cyclophellitol from D-xylose

8.4 Heterologous Expression in Fungi

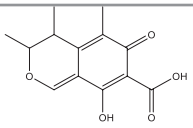
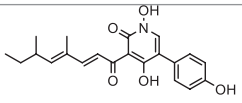
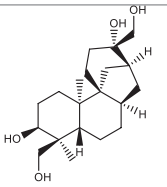
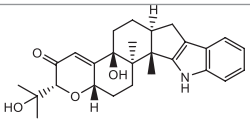
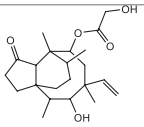
Heterologous expression is defined as expression of a gene in the host organism which naturally does not have that desired gene. Recombinant DNA technologies have been used in inserting foreign DNA in the host organism. To study the fungal synthetic biology, heterologous expression system has been used, as it not only allows one to study biosynthetic pathway or enzyme involved but also gives an idea about potential of fungi to be heterologous hosts (Kour et al. 2019). The first secondary metabolite attempted to express was Penicillin in 1990 by Smith and coworkers (Smith et al. 1990), and since then several studies reported that fungal biosynthetic enzyme is a valuable tool for secondary metabolite production. There are several studies regarding the expression of fungal enzyme in various hosts, from prokaryotes *E. coli* to single-celled eukaryotes and yeast (Kealey et al. 1998) to multicellular eukaryotes *Nicotiana tabacum* (Yalpani et al. 2001).One of the most promising heterologous expression system was reported in yeast *Pichia pastoris*, also known for its higher heterologous protein production. In addition to *E. coli* and yeast, filamentous fungi are often being used for heterologous expression of fungal secondary metabolites. Filamentous fungi are easy to grow and can be utilized for large-scale production. *Aspergillus* species are one of the most celebrated secondary hosts. Among filamentous fungi, *Aspergillus nidulans* is a model species and has been used to study gene cluster from other species (Cereghino and Cregg 2000). *A. oryzae* species is taxonomically close to model species *A. nidulans*. This species is of particular interest as a host for heterologous expression system because it has a long and safe history in food technology industry since it is being used by several countries for fermentation of cereals and also given the status of GRAS (generally recognised as safe) organism. Therefore, it can be utilized for producing secondary metabolites for human use (Barbesgaard et al. 1992).

Genetic manipulations such as selectable markers and promoters in *A. Oryzae* have been done using recombinant DNA technologies to ensure high level of expression of secondary metabolite in the organism (Yamada et al. 1997; Jin et al. 2004;Pahirulzaman et al. 2012), which in turn can increase the yield percentage of bioactive secondary metabolite. Pleuromutaline an antibiotic synthesized in *A. oryzae* by Bailey et al. (2016) is a diterpene compound naturally produced in *Clitopilus passeckerianus* and related species (Hartley et al. 2009). Table 8.4 describes the common secondary metabolite produced through heterologous mode of expression in the secondary host *A. oryzae* and its native host.

8.5 Conclusion and Future Prospects

Varieties of secondary metabolites are produced by fungal species; however, their production depends upon the growth condition. Compounds produced are of important chemical classes like alkaloids, terpenes, terpenoids, and sugar derivatives, for example, alliacol A, pasteurestins A and B, clavaric acid, helvolic acid, ergotamine,

Table 8.4 Structure, native host of secondary metabolite produced via heterologous mode of expression within the secondary host *Aspergillus oryzae*

Natural product	Native host	Structure	Function	References
Citrinin	*Monascus purpureus*		Antibacterial	Sakai et al. (2008)
Tenellin	*Beauveria bassiana*		Inhibitor of ATPase activity of erythrocyte membrane; iron chelator	Heneghan et al. (2010)
Aphidicolin	*Phoma betae*		Inhibitor of DNA polymerase α	Fujii et al. (2011)
Paxilline	*Penicillium paxilli*		Inhibitor of the high-conductance calcium-activated potassium channel; antibacterial	Tagami et al. (2013)
Pleuromutilin	*Clitopilus passeckerianus*		Antibacterial	Bailey et al. (2016)

psilocybin, meleagrin, cyclophellitol, etc. They are reported to have a variety of biological properties—antibacterial, anticarcinogenic, enzyme inhibitory, hepatoprotective, and others. However, at the laboratory level, the production of fungal secondary metabolite is very low; this leads to search alternative strategies to synthesize chemical compound in laboratory condition and at a higher rate of production. These objectives were somewhat achieved by chemical synthesis of compound. Moreover, total in vitro chemical synthesis does not always provide a way to produce chemical compounds at higher yield, however to overcome this problem, heterologous mode of producing secondary metabolite can be done. Heterologous expression of secondary metabolite is an effective strategies for describing cryptic gene, to achieve bioactive natural product clean background which aids to purification and downstreaming of natural product.

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Chapter 9
Fungal Community for Novel Secondary Metabolites

Enespa and Prem Chandra

9.1 Introduction

Fungal communities have a vitally important role in our routine life, whether positive or negative (De Vries and Shade 2013). They are origins of lifesaving and life-enhancing drugs, food additives, and aromas, but they also have the potential to contaminate our crops and food or to cause serious infections (Gerke and Braus 2014). Microbes such as fungi, bacteria, plants, and some insects produce secondary metabolites (Kusari et al. 2013). These natural products are low molecular weight molecules that, differing from primary metabolites, are not indispensable for the survival of the organism but confer an advantage in specific habitats or during changes in environmental conditions (Lange 2015). Various secondary metabolites possess biological activities that range from beneficial to harmful (Brandt and Mølgaard 2001).

Advantageous secondary metabolites (SMs) include antifungal agents such as caspofungin (Macheleidt et al. 2016), antibacterial agents such as penicillin, anticancer drugs such as taxol, immunosuppressive drugs such as cyclosporine, or cholesterol-lowering drugs such as lovastatin (Li and Vederas 2009). A growing problem is the amazing current and future increases in resistance against established antibiotics as was foretold by the WHO (Brown and Wright 2016). Antibiotic use in clinical medicine, stock breeding, and agriculture leads to the development of multiresistances, especially in daily applications where various known antibiotics are ineffective (Chang et al. 2015). Thus, the innovation of novel drugs is essential (Li and Pan 2014). Various species of fungi such as *Aspergillus niger* are used for the large-scale fermentation of citric acid and gluconic acid and are industrially exploited as enzymes, food additives, and medicinal drugs (Dhillon et al. 2011).

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The fungus *Aspergillus oryzae* is used in Asian cuisine for the fermentation of soybeans, saccharification of rice, and production of alcoholic drinks and rice vinegars (Murooka and Yamshita 2008), and the fungus *Monascus purpureus* is used for a natural food coloring (Mapari et al. 2010). In food preparation that uses fungi, information about obtainable secondary metabolite gene clusters becomes even more significant as potentially harmful clusters of gene might lurk in the genome and represent a risk of alcoholism (Takeda et al. 2014). Some mycotoxins are produced by various *Aspergillus* sp., followed by citrinin and patulin, which are produced by *Aspergillus* and *Penicillium* sp., and *Fusarium*-specific toxins such as zearalenone, but the harmful secondary metabolites such as aflatoxins are prominent (Gerke and Braus 2014).

The mycotoxin-producing fungi in crop contamination lead to more than 10% loss in the yield of agricultural crops globally, representing a massive economic problem (Savary et al. 2012), although the pathogenic fungal spores that are harmful for both plants and animals can also cause various diseases. Allergic reactions are also induced by inhalation of fungal spores (Douwes et al. 2003). *Aspergillus fumigatus*, *Aspergillus flavus*, and *Aspergillus terreus* cause infection and can lead to invasive aspergillosis, which can be life threatening in immunocompromised patients (Stevens et al. 2000). Communities of fungi have the potential to produce various secondary bioactive metabolites used as therapeutic agents against several diseases directly or indirectly (Kusari et al. 2012). The production of secondary metabolites from the plant host with therapeutic potential such as taxol, podophyllotoxin, deoxypodophyllotoxin (Zhao et al. 2011), camptothecin and structural analogues, azadirachtin, hypericin, and emodin by fungal communities has been discovered (Chagas et al. 2018). Fungal communities produce bioactive compounds that are not only important from the ecological aspect but also from a biochemical and molecular position, especially those exclusive to their host plants (Berg and Smalla 2009).

The production of excess known and novel bioactive secondary metabolites may occur when exploiting the fungal community, such as modifying the available culture and process. The compounds produced by fungal communities might be optimized using controlled fermentation conditions, possibly leading to a cost-effective, environmentally friendly, continuous, and reproducible yield on commercial scale-up (Chan et al. 2003). The reduction of secondary metabolite production on repeated subculturing in axenic monoculture conditions needs to be described to establish, restore, and sustain the in vitro biosynthetic potential of endophytes, one of the key challenges. The fact that nearly all efforts to obtain secondary metabolites from fungal communities have so far been made by classical methodology, under axenic monoculture conditions, increased this problem (Kusari et al. 2012).The renewal of known secondary metabolites led occasionally to mostly overlooking the collection of cryptic products that are not formed naturally under standard in vitro conditions (Bills et al. 2013). To imagine the aforesaid challenges, in this perspective the basic principles of chemical networking approaches of fungal communities with their host plants highlight forthcoming directions and the virtually unlimited possibilities

for discovery and the maintainable production of objective and not expected secondary metabolites exploiting fungal communities (Demain et al. 2017).

9.2 Collection and Detection Methods for Fungal Bioactive Compounds

The study of fungal metabolites has proceeded behind the study of other fungal metabolites because of scientific and organizational constraints (Morath et al. 2012). Moreover, the production of secondary metabolite (SM) production is bioactive (Stergiopoulos et al. 2013). The SM profiles fluctuate and depend entirely on the substrate, incubation period, nutrient media, temperature, and various environmental factors of given strains or species (López-González et al. 2015: Jurado et al. 2014). During the past half-century, there has been substantial progress on various compounds. The SMs of fungus determined by gas chromatography–mass spectrometry (GC-MS) and high performance liquid chromatography (HPLC) have been used recently because of their dominant separation and highly sensitive detection abilities (Turner et al. 2009). Tenax can be used for the concentration of headspace culture of solid adsorbent, followed by thermal desorption into the GC-MS (Bicchi et al. 2008). A library of mass spectra, database, or by comparative study of known standards of retention times and spectrum identified the SMs (Bino et al. 2004). In the headspace culture the volatile organic compounds adsorb or desorb by another method known as solid-phase micro-extraction (SPME) (David and Sandra 2007). This method decreases the time of preparation by combining extraction, introduction, and concentration into one step while increasing sensitivity over other extraction methods. Thus, this method has become popular recently (Hamelinck et al. 2005).

The living fungal cultures can be mechanized for headspace-SPME GC-MS by shortest profiling (Gao and Xu 2015). Novel volatile compounds cannot be determined by GC-MS, so this is one drawback. Simultaneous distillation extraction (SDE) of traditional methods such as vapor distillation and solvent extraction are used for the determination of secondary metabolites from *Penicillium roqueforti* and compared to the SPME method (Ridgway et al. 2010). Selected ion flow tube–mass spectrometry (SIFT-MS) in complex gas mixtures provides rapid broad-spectrum detection of trace secondary metabolites (Scotter et al. 2005). The production of secondary metabolites is detected from various species of fungi such as *Aspergillus*, *Candida*, *Mucor*, *Fusarium*, and *Cryptococcus* by the SIFT-MS technique (Morath et al. 2012). Proton transfer reaction–mass spectrometry (PTR-MS) and GC-MS instruments determine the profile of SMs released by *Xanthomonas* sp. The fungal SMs are quantified by using PTR-MS because it has fine detection ability and a fine-scale time response (Giannoukos et al. 2017). Moreover, examinations can be run without sample preparation, derivatization, or concentration in real time with the advantage of having sensitivities comparable to GC-MS (Hajslova et al. 2011).

This technique is also used for quantification of the SMs of *Muscodor albus* (Leelasuphakul et al. 2008). For further analysis and separation of the potential of secondary metabolites to identify innovative compounds produced by fungi, the sample is placed in a stainless steel column, then recovered and determined by nuclear magnetic resonance (NMR) spectroscopy (Strobel 2014). The "electronic nose" (E-nose) is an advanced technique used for bioactive compounds. An information processing unit with pattern recognition software and reference library is combined in the E-nose system by multisensory array (Carey et al. 2011). The SM production studies and results from examining numerous microbes and diversified communities of soil microbes of soil by several techniques are listed in Table 9.1.

9.3 Fungal Bioactive Compounds as Sources of Secondary Metabolites

For exploiting the bioactive metabolite compounds, fungi are the key resources (Harvey 2008). Between the fungi, biologically active metabolites are screened from the endophytes (Strobel and Daisy 2003). Without causing any disease symptoms, endophytic fungi inhabit within their host plants (Schulz et al. 2002). The low molecular weight compounds not required for growth in pure culture known as secondary metabolites are manufactured as a revision for specific functions in nature (Bérdy 2005). In the interactions of numerous metabolites between fungi and their plant hosts, such as signalling, defence, and instructions of the symbiosis, the SMs have a vital role in vivo (Tanaka et al. 2006).

Diverse classes of chemical substances such as steroids, xanthones, phenols, isocoumarines, perylene derivatives, quinones, furandiones, terpenoids, depsipeptides, and cytochalasines have been isolated from endophytic fungi (Nisa et al. 2015; Rana et al. 2018a; Suman et al. 2016; Yadav et al. 2018). Using non-ribosomal protein synthesis, such substances are synthesized through the polyketide pathway. A complex of *Burkholderia cepacia* non-ribosomal peptide-synthesized toxin is hemolytic and required for full virulence (Thomson and Dennis 2012). The various novel chemical structures produced by endophytes (51%) are significantly higher than the soil fungus (38%), as revealed from a literature survey suggesting that these habitually discounted endophytes are the novel source of bioactive secondary metabolites (Gnansounou et al. 2017). Special substances such as secondary metabolites are produced and in return demand nutrition. They are known to prevent the host from successfully attacking fungi and pests (Kaul et al. 2012; Nisa et al. 2015). With more resistance to nematodes, insects, and livestock, the fungal communities synthesize an array of metabolites for plants (Bassman 2004; Kaul et al. 2012).

Because of the production of phytohormones with specific endophytes inhabiting them, plants can grow faster and become so economical that they predominate in a specific environment (Herms and Mattson 1992; Rana et al. 2016, 2018a, b). The chemical compounds or secondary metabolites that are synthesized inside plants by the endophytes are associated with medicinal plants and can be exploited

Table 9.1 Methods applied for the detection of bioactive compounds from different fungal species

Methods	Organisms investigated	Habitat/ cultivation media	Bioactive compounds found	References
GC-MS	*Aspergillus* spp., *Cladosporium cladosporioides*, *Penicillium* spp.	Dichloran glycerol agar	Diverse bioactive compounds	El Sheikha et al. (2018)
GC-MS	Fungal community	Hyperthermic, hypersaline soils	Diverse bioactive compounds	Hock et al. (2018)
GC-MS	*Muscodor albus*	Modified minimal medium	Esters, alcohols, lipids, ketones	Enespa and Chandra (2017)
PTR-MS/ PTRTOF-MS	Fungal community	Temperate soil under different compost load	Diverse bioactive compounds	Enespa and Chandra (2017)
GC-MS	*Aspergillus fumigatus*	Modified minimal medium	Dimethyl sulfide (DMS), dimethyl disulfide (DMDS), 2,5-dimethylpyrazine (2,5-DMP), 1-undecene, 2-nonanone, 2-undecanone, and 2 aminoacetophenone (2-AAP)	Briard et al. (2016)
PTR-ToF MS, GC-MS, Electronic nose (e-nose) analysis	*Erwinia amylovora, Pseudomonas syringae* pv. *syringae*	Rooted plantlets, Murashige and Skoog (MS) medium	2-Ethoxy-2-methyl propane, 2,4,4-trimethyl-1-pentene and 2-methyl-furan	Cellini et al. (2016)
GC-MS	Fungal community	Hyperthermic, hypersaline soils	Diverse bioactive compounds	Miller et al. (2015)
GC-MS	*Muscodor albus* E-6 Endophytic fungus of *Guazuma ulmifolia*	Cultivated on potato dextrose agar (PDA)	Diverse bioactive compounds	Saxena et al. (2015)
GC-MSD (mass selective detector)	Fungal community	Orange waste	Monoterpenes, isoprene, other bioactive compounds	Li et al. (2012)
GC-MS	*Aspergillus* spp., *Cladosporium cladosporioides*, *Penicillium* spp.	Dichloran glycerol agar	Diverse bioactive compounds	Beck (2012)

(continued)

Table 9.1 (continued)

Methods	Organisms investigated	Habitat/ cultivation media	Bioactive compounds found	References
PTR-MS	*Shigella flexneri, Candida tropicalis*	Complex media	Diverse VOCs, several unidentified and some identified compounds of low molecular weight <150 μ	Effmert et al. (2012)
PTR-MS	Fungal community	Organic waste	Various bioactive compounds	Morath et al. (2012)
GC-MS	*Hypholoma fasciculare Resinicium bicolor*, wood-decaying fungi	Cultivated on malt broth	Diverse bioactive compounds	Sasidharan et al. (2011)
GC-MS/ growth inhibition of bacterial cultures	*Fusarium oxysporum* strain MSA 35	Agar (as described in experimental procedures)	Diverse bioactive compounds	Kai et al. (2010)
GC-MS	Fungal community	Different Mediterranean soils	Diverse bioactive compounds	Ens et al. (2009)
GC-MS	Fungal community	Different Mediterranean soils	Diverse bioactive compounds	Leff and Fierer (2008)
GC-MS	*Fusarium* spp.	MEA and PDA	Sesquiterpenes, mainly trichodiene	Perkowski et al. (2008)
GC-MS	*Muscodor albus* E-6 Endophytic fungus of *Guazuma ulmifolia*	Cultivated on PDA	Diverse bioactive compounds	Strobel et al. (2007)
GC-MS	*Fusarium* spp.	MEA and PDA	Sesquiterpenes, mainly trichodiene	Jeleń and Grabarkiewicz-Szczęsna (2005)
GC-MS	*Muscodor albus*	Endophytic fungus of *Cinnamonum*, cultivated on PDA	Diverse bioactive compounds	Ezra et al. (2004)
GC-MS	*Muscodor albus*	Endophytic fungus of *Cinnamonum*, cultivated on PDA	Diverse bioactive compounds	Stinson et al. (2003)
GC-MS	*Sclerotinia minor*, *S. sclerotium*, *S. rolfsii*	Lettuce and bean isolates, cultivated on PDA	Diverse bioactive compounds	Harvey and Sams (2000)

for curing many diseases (Compant et al. 2005; Strobel and Daisy 2003). The bioactive metabolites in a large number of endophytic fungi belong to diverse structural groups known as alkaloids, steroids, terpenoids, peptides, polyketones, flavonoids, quinols, phenols, xanthones, chinones, isocumarines, benzopyranones, tetralones, cytochalasines, perylene derivatives, furandiones, depsipeptides, and enniatines that have been extracted, characterized, and isolated (Tenguria et al. 2011). The novel structural groups represented by several of these are palmarumycins and a new benzopyroanone (Schulz et al. 2002). The fungi-produced secondary metabolites may vary with the biotope in which it grows and adopted, which varied with both habitat and substrate such as the manufacture of cyclosporine A, enchinocandin B, papulacandins, and verrucarins (de Carvalho et al. 2015). Screenings of natural products are the source of endophytic fungi, and in optimizing the search for secondary metabolites of new bioactive chemical compounds, it is relevant to consider that a fungus that synthesizes the SMs may resemble its particular ecological niche and metabolic interactions, which continue between the fungus and plant to enhance the production of secondary metabolites (Bérdy 2005; Cragg and Newman 2013).

In addition to being alternative sources for secondary metabolites known from plants, endophytes accumulate a wealth of other biologically active and structurally diverse natural products that are unprecedented in nature (Nisa et al. 2015; Proksch et al. 2010) It is now generally accepted that endophytes represent an important and largely untapped reservoir of unique chemical structures that have been modified through evolution and are believed to be involved in host plant protection and communication (Farrar et al. 2014). The fungal endophytes are known to release metabolites that mimic the structure and function of host compounds and produce plant growth hormones such as gibberellins (Hyde and Soytong 2008). A wide range of biological activities such as those of the antimicrobial agent hypericin and acetylcholinesterase inhibitor huperzine A are plant-associated secondary metabolites produced by prolific endophytes (Xiong et al. 2013), the antitumour agents taxol (Cai et al. 2015). Endophytes of bioprospecting offer promise to determine natural products with therapeutic value, which has increased attention from microbiologists, ecologists, agronomists, and chemists (Qin et al. 2011).

The endophytic fungi have great interest as potential producers of novel, biologically active products (Yadav 2018; Yadav et al. 2017; Strobel and Daisy 2003). The distribution of endophytic mycoflora differs with the host, known as an important component of biodiversity and also considered as endophytes (Khan et al. 2010). Globally, the necessity of new pharmaceutical products such as antibiotics, agrochemicals, and chemotherapeutic agents to manage the rising medicinal and ecological problems faced by mankind has increased interest in research on fungal community chemistry (Paladini et al. 2015). The mangrove plant *Rhizophora annamalayana* is the host of an endophytic fungus isolated and characterized for the production of taxol (Elavarasi et al. 2012). The extraction of secondary metabolite taxol is accomplished with ethyl acetate and characterized by chromatographic and spectrometric analysis (Fraser et al. 2000). The infrared (IR) spectrum values confirmed terpenoid functional groups and the violet-red represented by a thin-layer chromatographic plate (Milgram et al. 2007). In the leaf of *Cynodon dactylon*, an endophytic fungus, *Aspergillus fumigatus* CY018, was recognised for the first time (Liu et al. 2004).

The endophytic fungus *Taxomyces andreanae*, in producing paclitaxel from the yew plant *Taxus brevifolia*, set the stage for a more inclusive investigation of other species and other plants for the presence of paclitaxel-manufacturing endophytes (Pu et al. 2013), so as to apply this to developing the production of this pharmacologically important drug (Cohen 2002). The multi-billion dollar anticancer compound paclitaxel, produced by the yew plant (Chabner and Roberts 2005), has action against a broad range of tumour types (Kulbe et al. 2004), including breast, ovarian, lung, and head and neck cancers, as well as progressive forms of Kaposi's sarcoma (Vihinen and Kähäri 2002).

Production of loline alkaloids occurs by infection of grasses with endophytes which display restrictive and toxic effects towards herbivorous invertebrates and vertebrates and thus form a possible complex in protection of endophyte-infected grasses against herbivores (Saikkonen et al. 1998; Schardl et al. 2004). The three new antimicrobial metabolites and the indole-3-acetic acid (IAA) plant hormone were analysed from the culture of *Colletotrichum* sp., an endophyte isolated from inside the stem of *Artemisia annua* (Lu et al. 2000; Tan and Zou 2001). The isolation and characterization of various other chemical compounds such as ergosterol (I), 3b,5a,6b-trihydroxyergosta-7,2,2-diene (II), 3b-hydroxy-ergosta-5-ene (III), 3-oxo-ergosta-4,6,8 (14), 2,2-tetraene (IV), 3b-hydroxy-5a,8a-epidioxy-ergosta-6,2,2-diene (V), 3b-hydroxy-5a,8a-epidioxy-ergosta-6,9 (11), 2,2-triene (VI), and 3-oxoergosta-4-ene (VII) was also completed from the culture of a fungal community (Nisa et al. 2015). The growth inhibition of tested bacteria such as *Staphylococcus aureus, Bacillus subtilis, Pseudomonas* sp., and *Sarcina lutea* takes place by 1e3 and IIIeV chemical compounds (Son et al. 2016). Three species represent positive hits by screening of molecular markers and have the capability of producing taxol, which was authenticated by HPLC-MS. Among these three taxol-producing fungi, the yield of taxol was greater in *Guignardia mangiferae* HAA11 720 ng/l compared with *Fusarium proliferatum* HBA29 (240 ng/l) and *Colletotrichum gloeosporioides* TA67 (120 ng/ l), the fungal strain possessing antimicrobial activity (Liu et al. 2009; Chaturvedi 2015) (Table 9.2).

9.4 Antifungal Bioactive Compounds from a Fungal Community

Pathogenic fungi are controlled by secondary metabolites of some biocontrol fungi (Rohlfs and Churchill 2011). The mycoparasitism, nutrient competition, and secretion of other inhibitory compounds and hydrolytic enzymes by the various species of *Trichoderma* control the soil-borne fungal pathogens by various mechanisms (Benítez et al. 2004). The inhibition of growth and production of proteins from a wood-rotting basidiomycete *Serpula lacrymans* takes place from secondary metabolites secreted from *Trichoderma viride* and *T. aureoviride* (Schoeman et al. 1999). However, *T. pseudokoningii* showed no effect in any of the *Serpula lacrymans* isolate tests (Wheatley 2002; Bitas et al. 2013). The secondary metabolites secreted by

Table 9.2 Novel secondary metabolites from endophytic fungi

Fungal species	Origin	Secondary metabolites	Reported activities	References
Alternaria alternata	Terrestrial, grapevine leaves	9-Methoxy CPT	Antifungal, anticancer	Chakravarty and Gaur (2018)
Alternaria alternata RSF-6 L	Terrestrial, *Brassica napus*	Indole-3-acetic acid (IAA)	Antifungal, PGP	Yan et al. (2018)
Actinoallomurus fulvus	Terrestrial, *Capsicum frutescens*	Actinoallolides	Anti-trypanosomal	Nandi et al. (2019)
Penicillium manginii	Terrestrial, *Panax notoginseng*	Duclauxamide	Cytotoxicity	Bedi et al. (2018)
Cytospora sp.	Terrestrial, *Conocarpus erecta*	Cytoskyrins	BIA activity	Gao et al. (2018)
Periconia sp.	Terrestrial, *Annona muricata*	Pericoannosin	Anti-HIV	Gao et al. (2018)
Peyronellaea coffeae-arabicae	Terrestrial, *Pritchardia lowreyana*	Peyronellins	Cytotoxicity	Gao et al. (2018)
Mucor irregularis	Marine, *Rhizophora stylosa*	Rhizovarins	Cytotoxicity	Zhou and Xu (2018)
Rhizoctonia solani	Terrestrial, *Cyperus rotundus*	Solanioic acid	Antimicrobial	Dissanayake et al. (2016)
Fusarium sp. JZ-Z6	Terrestrial, *Fritillaria unibracteata*	Gallic acid	Antioxidant, anticancer	Pan et al. (2017)
Penicillium sp.	Terrestrial, *Catharanthus roseus*	Citreoviripyrone	Cytotoxicity	Jiménez-Romero et al. (2017)
Arthrinium sp. 0042	*Aquilaria subintegra*	oxo-Agarospirol	Antioxidant	Monggoot et al. (2017)
Penicillium brocae	Marine	Spirobrocazines	Antibacterial, cytotoxicity	Muharini et al. (2017)
Campylocarpon sp.	Marine, *Sonneratia caseolaris*	Campyridones	Cytotoxicity	Zhu et al. (2016)
Pestalotiopsis sp.	Marine, *Rhizophora mucronata*	Pestalotiopens	Antimicrobial	Xu (2015)
Paecilomyces variotii	Marine	Varioxepine	Antimicrobial	Zhang et al. (2015)
Trichoderma gamsii	Terrestrial, *Panax notoginseng*	Trichodermone	Cytotoxicity	Ding et al. (2014)
Paecilomyces variotii	Marine	Varioxepine	Antimicrobial	Meng et al. (2014)
Aspergillus sp.	Marine	Asperterpenols	Acetylcholinesterase inhibition	Xiao et al. (2013)

(continued)

Table 9.2 (continued)

Fungal species	Origin	Secondary metabolites	Reported activities	References
Aspergillus versicolor	Marine, green alga *Codium fragile*	Aspeverin	Marine plant growth inhibition	Ji et al. (2013)
Fusarium sp.	Terrestrial, *Melia azedarach*	Fusarimine	Antifungal	Gao et al. (2013)
Pestalotiopsis fici	Terrestrial	Chloropupukean olides	Cytotoxicity	Ebrahim et al. (2012)
Pestalotiopsis sp.	Terrestrial, *Clavaroids* sp.	Torreyanic acid analogue	Antifungal	Gutierrez et al. (2012)
Pestalotiopsis virgatula	Terrestrial, *Terminalia chebula*	Pestalospiranes	Antimicribial	Kesting et al. (2011)
Chalara alabamensis	Terrestrial, *Asterogyne martiana*	Asterogynins	Antimicribial	Rosa et al. (2013)
Pestalotiopsis sp.	Terrestrial, clavaroid species	Torreyanic acid analogue	Antibacterial	Zou et al. (2011)
Microsphaeropsis sp.	Terrestrial, *Lycium intricatum*	Microsphaerops ones	Antibacterial	Yang and Li (2011)
Pestalotiopsis fici	Terrestrial	Chloropestolide	Anti-HIV, cytotoxicity	Liu et al. (2010)
Nodulisporium sp.	Marine, alga	Noduliprevenone	Cytotoxicity	Greve et al. (2010)
Phaeosphaeria avenaria	Terrestrial	Phaeosphaeride	Inhibiting STAT3 activity	Weber (2009)
Phaeosphaeria avenaria	Terrestrial	Phaeosphaeride	Inhibiting STAT3 activity	Schlingmann et al. (2007)
Cytospora sp.	Terrestrial, *Conocarpus erecta*	Cytoskyrins	BIA activity	Gunatilaka (2006)
Cryptosporiopsis cf. *quercina*	Terrestrial, *Triptergyium wilfordii*	Cryptocin	Antifungal, Antibacterial	Strobel et al. (2005)
Fusarium pallidoroseum	Terrestrial	Apicidins	Antiprotozoal, anticancer	Somei and Yamada (2004)
Cryptosporiopsis cf. *quercina*	Terrestrial, *Triptergyium wilfordii*	Cryptocin	Antifungal	Strobel and Daisy (2003)
Pestalotiopsis sp.	Marine, *Rhizophora mucronata*	Pestalotiopens	Antimicrobial	Schulz et al. (1995)

various isolates of three *Trichoderma* spp. exhibited a degree of growth inhibition against a soil-borne fungal pathogen *Fusarium oxysporum* f. sp. *ciceris* that causes chickpea wilt disease (Gopalakrishnan et al. 2011). *F. oxysporum* strain MSA35 secreted secondary metabolites that enhanced the growth of lettuce plants and in the presence of ectosymbiotic bacteria also released the secondary metabolites that inhibit the growth of pathogenic strains of *F. oxysporum* (Enespa and Chandra 2017).

Antifungal metabolites revealed over time by the fermentation of dung-inhabiting fungi, or other compounds, are contrary to plant pathogenic fungi (Fu et al. 2012). The antagonistic features displayed by *Sordaria fimicola* against soil-borne pathogenic fungi such as *Pythium aphanidermatum* and *Dematophora necatrix* caused disease against the plant (Sarrocco 2016). The isolation of *S. fimicola* from wheat and ryegrass roots could reduce the size of these masses after inoculation with the take-all fungus (*Gaeumannomyces graminis* var. *tritici*) (Zhang et al. 2017). The submerged culture of *Coprinus heptemerus*, a basidiomycete, secreted seven diterpenoids, named heptemerones A to G that previously were not known to produce secondary metabolites (Molitor et al. 2012; Pettit et al. 2009). The chemical compounds were purified and tested for their antifungal activities, which inhibited the fungal germination, but this was highly dependent on the composition of the assay medium (Lavermicocca et al. 2000).

Four of the antifungal compounds exhibited plant protective activity in a leaf segment assay using *Magnaporthe grisea* as the pathogen (Kettering et al. 2005). *Podospora decipiens*, *Podospora curvicola*, and *Podospordaria tulasnei* have exposed antifungal activity by secondary metabolites against *Fusarium verticillioides*, *Aspergillus flavus*, and *F. verticillioides* and *Fusarium fujikuroi* (Cardwell et al. 2000), respectively. In agriculture, the demand is increasing for new antifungal compounds in the continuous search for new effective and natural fungicides for use against plant pathogens in integrated pest management (Dayan et al. 2009; Oerke 2006).

The academic institutions and agrochemical industries have been manufacturing new crop protection agents of microbial origin, which are safer for both the environment and consumers and more effective than the existing agents (Chandler et al. 2008). The naturally derived active pesticide ingredients are used in line with EC within the structure of achieving the sustainable use of pesticides by reducing the risk and impacts of their use on human health and the environment, and encouraging the use of integrated pest management and of unconventional techniques (Khater 2012). In this perspective, the fungal communities represent an uncultivated pool of bioactive metabolites, with chemical innovations that can be tested and further developed as active constituents in plant protection products (Lorenz and Eck 2005). Secondary metabolites-secreted antifungals by fungi against phytopathogenic fungi are given in Table 9.3 and Fig. 9.1.

9.5 Antibacterial Bioactive Compounds from Fungal Community

The fungal communities produced secondary metabolites that are larger than those of any other microorganism (Dean et al. 2005). These microorganisms occur in high frequency and are isolated from plants (Schippers et al. 1987). Numerous fungal genera seem to have a higher frequency of isolation and therefore a comparatively greater chance of an antibacterial substance being discovered in the species for

Table 9.3 Fungal bioactive compounds secreted by fungi against phytopathogenic fungi

Fungal antagonists	Bioactive compounds	Effects	Pathogenic fungi	References
Candida albicans	Farnesol	Inhibition of mycelial development, Apoptosis in altered morphology and reduced fitness	*Aspergillus nidulans*, *Fusarium graminearum*	Conrad et al. (2018)
Irpex lacteus, *Hypoxylon anthochroum* Blaci	Benzothiazole, cyclohexanol, *n*-decanal, dimethyl trisulfide, 2-ethyl-1-hexanol	Growth inhibition	*Alternaria solani*, *Botrytis cinerea*	Gao et al. (2017)
H. anthochroum Blaci	2-Methyl-5-(1-methylethyl)-bicyclohexan-2-ol, 2, 6-dimethyl-2, 4,6-octatriene	Inhibiting effect on growth of oomycetes	*Pythium ultimum*, *Phytophthora capsici*, *Alternaria solani*, *Fusarium oxysporum*	Ulloa-Benıtez et al. (2016)
Hypsizygus marmoreus	2-Methylpropanoic acid 2,2-dimethyl-1-(2-hydroxy-1- methylethyl) propyl ester	Inhibitory effect against conidial germination	*A. brassicicola (O-264)*	Oka et al. (2015)
Phomopsis sp.	Sabinene; isoamyl alcohol; 2-methyl propanol; 2-propanone	Worked as antibiotic effects	*Pythium*, *Phytophthora*, *Sclerotinia*, *Rhizoctonia*, *Fusarium*, *Botrytis*, *Verticillium*, *Colletotrichum*	Lee (2015)
Cladosporium cladosporioides CL-1	α-Pinene, β-caryophyllene, tetrahydro-2,2,5,5 tetramethylfuran, dehydroaromadendrene, sativene	Growth inhibition of mycelium	*Pseudomonas syringae*	Kamchiswamy et al. (2015)
Ampelomyces sp.	*m*-Cresol	Inhibition of mycelial growth	*Pseudomonas syringae* pv.	Naznin et al. (2014)

(continued)

Table 9.3 (continued)

Fungal antagonists	Bioactive compounds	Effects	Pathogenic fungi	References
Mycoleptodonoides aitchisonii	1-Phenyl-3-pentanone	Strongly inhibited the mycelial growth, spore germination	*Alternaria alternata*, *A. brassicicola*, *A. brassicae*, *Colletotrichum orbiculare*, *Corynespora cassiicola*	Nishino et al. (2013)
Epichloe typhina	Sesquiterpenes, chokols A–G	Fungitoxic	*Cladosporium phlei*	Kumar and Kaushik (2012)
Phoma sp.	Series of sesquiterpenoids, some alcohols, reduced naphthalene derivatives	Antifungal and fuel properties; some of the test organisms with the greatest sensitivity	*Verticillium*, *Ceratocystis*, *Cercospora*, *Sclerotinia*	Strobel et al. (2011)
Saccharomyces cerevisiae CR-1	3-Methylbutan-1-ol, 2-methylbutan-1-ol, 2-phenylethanol, ethyl acetate, ethyloctanoate	Inhibits vegetative development	*Guignardia citricarpa*	Fialho et al. (2010)
Saccharomyces cerevisiae	Ethyl acetate, 2-methylbutan-1-ol, 3-methylbutan-1-ol, 2-phenylethanol, ethyloctanoate	Growth inhibition	*G. citricarpa*	Verginer et al. (2010)
Trichoderma viride, *Trichoderma harzianum*	6-Pentyl-α-pyrone, β-1-3, glucanases	Phytotoxicity during seedling formation, seedling blight suppression	*Fusarium oxysporum*, *Rhizoctonia solani* (Israel), *Pythium ultimum* (USA)	El-Hasan and Buchenauer (2009)
Candida albicans	Farnesol	Inhibition of mycelial growth, apoptosis in altered morphology and reduced fitness	*Aspergillus nidulans*, *Fusarium graminearum*	Leveau and Preston (2008)

(continued)

Table 9.3 (continued)

Fungal antagonists	Bioactive compounds	Effects	Pathogenic fungi	References
Irpex lacteus	5-Pentyl-2-furaldehyde	Suppressed the growth	*F. oxysporum* f. sp. *lycopersici*, *Bulmeria graminis*, *Fusarium oxysporum*, *Colletotrichum Fragaria*, *Botrytis cinerea*	Koitabashi (2005)
Muscodor albus	Ethyl acetate, propanoic acid, 2-methyl-methyl ester, ethanol, acetic acid, 2-methylpropyl ester, propanoic acid, 2-methyl-butyl ester, 1-butanol, 2-methyl	Inhibited the growth of fungi	*Pythium ultimum*, *Phytophthora cinnamomi*, *Rhizoctonia solani*, *Ustilago hordei*, *Stagnospora nodorum*, *Sclerotinia sclerotiorum*, *Aspergillus fumigatus*, *Verticillium dahliae*, *Cercospora beticola*, *Xilaria* sp.	Ezra et al. (2004)

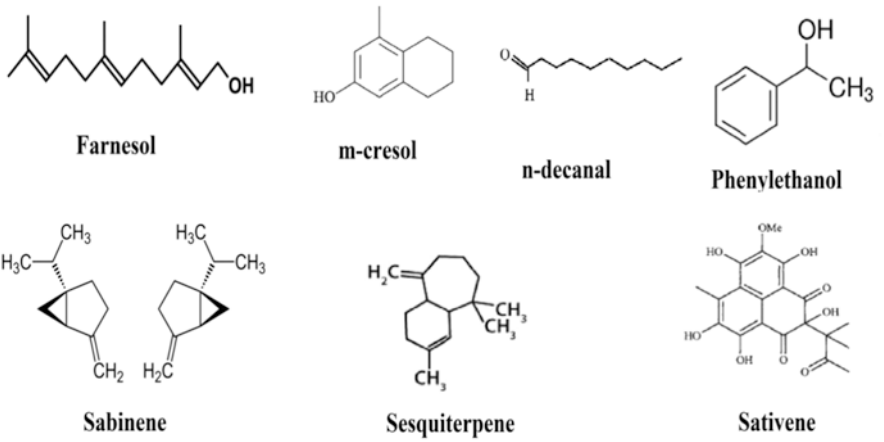

Fig. 9.1 Structural formulas of some of the antifungal bioactive compounds produced by fungal community

similar reasons (Radić and Štrukelj 2012). The various new secondary metabolites isolated and extracted from the endophytic fungus *Alternaria* sp. are 10-oxo-10*H*-phenaleno [1,2,3-de] chromene-2-carboxylic acids, xanalteric acids I and II (Fig. 9.2), and 11 other chemical compounds (Firáková et al. 2007). This fungus was isolated from the mangrove plant *Sonneratia alba* and exhibited weak antibacterial activity against *Staphylococcus aureus* (Debbab et al. 2010). The broad antimicrobial activity against several resistant pathogens with minimum inhibitory concentration (MIC) values in the range of 31.25–125 g/ml exhibited altenusin (Fig. 9.2) (Deshmukh et al. 2015).

Local people used *Aspergillus* sp. HAB10R12 for peptic ulcer and postpartum care was isolated from the root of *Garcinia scortechinii* (Ramasamy et al. 2010). The host plant *G. scortechinii* released xanthones that inhibit methicillin-resistant *Staphylococcus aureus* (MRSA) (Lin et al. 2017; Alurappa et al. 2018). *Aspergillus* sp. HAB10R12 showed antibacterial effect similar to that of the control antibiotics against *Micrococcus luteus* and *S. aureus* and significantly superior to gentamicin against *Bacillus subtilis* and *Escherichia coli* and cephalexin against *B. subtilis* (Ip et al. 2006). The naphthaquinone javanicin was highly functionalized (Fig. 9.2), with capable antibacterial activity, from an endophytic *Chloridium* sp. that was isolated from the surface-treated root tissues of *Azadirachta indica* (Kharwar et al. 2009).

Javanicin was active against *E. coli* and *Bacillus* sp. in the antibacterial test at a higher MIC value of 40 g/ml (Güllüce et al. 2003). This result could be an indicator of the selective antibacterial activity of javanicin, but it should be confirmed with additional testing (Rios and Recio 2005). The *Colletotrichum gloeosporioides* fungus, isolated from the medicinal plant *Vitex negundo* L., and three different extracts of hexane, ethyl acetate, and methanol were screened for their antibacterial activity

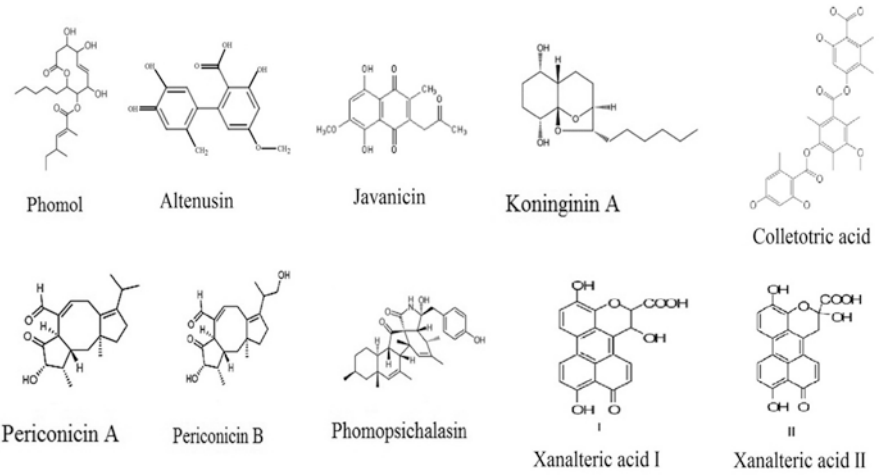

Fig. 9.2 Structural formulas of some of the antibacterial bioactive compounds produced by fungal community

against methicillin-, penicillin-, and vancomycin-resistant clinical strains of *S. aureus* (Arivudainambi et al. 2011). The same endophytic fungus isolated from the stem of *Artemisia mongolica* showed on antimicrobial bioassay that colletotric acid (Fig. 9.2), isolated from the culture liquid, was inhibitory to the bacteria *B. subtilis*, *S. aureus*, and *Sarcina lutea* (Darabpour et al. 2012).

In the same way, the metabolites released from *Colletotrichum* sp., an endophytic fungus isolated from *Artemisia annua*, had strong antimicrobial action against the bacteria *B. subtilis*, *S. aureus*, *Sarcina lutea*, and *Pseudomonas* sp. (Alurappa et al. 2018). *Colletotrichum* sp. was also isolated from another source such as healthy tissues of *Lippia sidoides*, a medicinal plant used as an antiseptic (de Siqueira et al. 2011). The endophytic fungus *Colletotrichum gloeosporioides* isolated from *Alternaria alternata*, *Guignardia biwelli*, and *Phomopsis archeri* shows antimicrobial assay only on solid medium (Barbieri et al. 2014). The plant parts of *Garcinia mangostana* released metabolites similar to the activity of their particular hosts, and a screening of the antibacterial activity of endophytic fungi isolated from surface-pasteurized leaves and small branches of *Garcinia mangostana* was conducted (Carvalho et al. 2016). The short branches of *Taxus cuspidata* inhabited an endophytic fungus *Periconia* sp., and secreted fusicoccane diterpenes, named periconicins A and B (Fig. 9.2) (Zaiyou et al. 2017).

The ethyl acetate chemical was used for the purification of these compounds and was active in antibacterial assays (Septama and Panichayupakaranant 2015). Periconicin A compounds demonstrated significant antibacterial activity against *B. subtilis*, *S. aureus*, *Klebsiella pneumoniae*, and *Salmonella typhimurium* with MIC in the range of 3.12–12.5 g/ml, in contrast to gentamicin, with MIC in the range of 1.56–12.5 g/ml. Periconicin B displayed different antibacterial activity against the same strains of bacteria with MIC in the range of 25–50 g/ml (Heitefuss 2011). *Phomopsis* sp., an endophytic fungus that secretes a metabolite known as phomopsichalasin represents the first cytochalasin-type compound with a three-ring system replacing the cytochalasin macrolide ring (Fig. 9.2).

Disk diffusion assays against *B. subtilis* (12-mm zone of inhibition) and *S. aureus* (8-mm zone of inhibition) showed antimicrobial activity by the secreted metabolites (Clay 1988). Phomol, known as a novel antibiotic, was isolated from the fermentation broth of *Phomopsis* sp. strain E02018, which secreted a novel antibiotic known as phomol secreted by fermentation broth in the course of a screening of endophytic fungi from the medicinal plant *Erythrina* (Cowan 1999) (Fig. 9.2). However, it showed moderate antibacterial activity against *Arthrobacter citreus*, *Corynebacterium insidiosum*, and *Pseudomonas fluorescens* in the serial dilution assay and was not active against *E. coli* or *B. subtilis* (Munaganti et al. 2016). Helvolic acid is a significant component that exhibited the strongest antibacterial activity against *E. coli*, *B. subtilis*, *S. aureus*, and *S. haemolyticus*, with MIC values of 3.13, 3.13, 50, and 6.25 g/ml, respectively, which was isolated from the endophytic fungus *Pichia guilliermondii* and evaluated by microdilution colorimetric activity (Gómez-Rivera et al. 2018).

Panax notoginseng, a herbal plant inhabiting the PRE-5 strain and which is identified as *Trichoderma ovalisporum*, secreted koninginin A, (E)-2,3-dihydroxypropyl

octadec-9-enoate, shikimic acid, cytosine ribonucleoside, and a compound considered to be adenine ribonucleoside from the culture broth (Fig. 9.2). Also, strain PRE-5 showed antibacterial activity against *S. aureus*, *B. cereus*, *M. luteus*, and *E. coli* (Dang et al. 2010).The culture extracts of the endophytic fungus *Xylaria* sp. YX-28, which is isolated from *Ginkgo biloba* L., was identified as 7-amino-4-methylcoumarin (Liu et al. 2008; Karaman et al. 2003). Determination of the antimicrobial activity of this chemical compound was observed by MICs and the agar-well diffusion method. The fungal community displayed strong antibacterial activity against pathogenic bacteria by all the secondary metabolites (Table 9.4).

9.6 Novel Approach to Obtaining Novel Bioactive Secondary Metabolites

Mutation, genetic manipulation, and cultural condition optimisation can improve the production of metabolites quantitatively and qualitatively (Hu et al. 2008). For the discovery of new metabolites and their biosynthetic pathways, the mutational approach is useful (Li and Vederas 2009).The generation of distinct phenotypes after analysis of mutants results from random mutagenesis, which is a powerful methodology to identify the essential factors for biological processes (Fiehn et al. 2000). For basic research and practical applications this self-assured genetic method is very important (Eisenstein 1990). A particularly increased sequence allowed by NGS techniques reduced the costs, thus qualifying the genomes of the mutant to be sequenced to identify affected genes (Meldrum et al. 2011).

Mutation identification strategies through whole-genome sequencing have been used for several model organisms, such as *Neurospora crassa* (Baird et al. 2008; Borkovich et al. 2004), with the premise that it is a efficient and rapid means to discover the mutations that are responsible for specific phenotypes (Letai et al. 1992). For survival, fungal communities must adapt to environmental stress, and a deeper understanding of the regulation and evolution of fungal stress response systems may lead to improved novel antifungal drugs and technologies (Frey-Klett et al. 2011).

Infrequently, the observation of a metabolic profile under standard fermentation does not reflect the number of anticipated biosynthesis genes of microorganisms, in that some loci remain silent (Knight et al. 2003). Because a reservoir of potentially bioactive compounds represents cryptic gene clusters, cryptic natural products strategies have been designed by triggering the biosynthetic pathways (Scherlach and Hertweck 2009). The transcription factors that mediated the fungal response to environmental cues such as nutrient availability, pH, light, and both biotic and abiotic stress are regulated by their secondary metabolites (Reverberi et al. 2010). To collect novel metabolites, the metabolic pathways of fungi are changed by the fermentation pathway (Papagianni 2004). By the addition of chromatin-modulating agents such as histone deacetylase or DNA methyl transferase inhibitors to fungal

Table 9.4 Fungal community producing metabolites with antibacterial activity

Endophytic fungal strain	Host plant(s) (family), plant part or tissue	Habitat of the host plant	Crude extract/isolated metabolite	Test bacteria	Type of test	Reference
49 endophytic fungi: The most active of the isolated strains: *Fusarium oxysporum* strain YNDC05 *Fusarium oxysporum* strain YNDC11 *Dictyosporium*	*Dracaena cochinchinensis* (Lour.) S.C. Chen (Asparagaceae); Ns *Dracaena cambodiana* Pierre ex Gagnep (Asparagaceae); ns	Yunnan Province, Hainan Province and Beijing, China	Ethanol extract of culture broth	*B. subtilis* (No. 1.0088), *S. aureus* (No.1.0089), *E. coli* (No. 1.2385)	Agar well diffusion method	Zheng et al. (2017)
Acremonium sp., *Diaporthe* sp., *Hypoxylon* sp., *Pestalotiopsis* sp., *Phomopsis* sp., *Xylaria* sp.	*Aegiceras corniculatum*, *Avicennia alba*, *Avicennia officinalis, Bruguiera gymnorrhiza*, *Bruguiera parviflora*	Mangrove areas in the south of Thailand in Satun, Songkhla, Surat Thani and Trang Provinces	Ethyl acetate crude extract of culture medium Ethyl acetate and hexane extract of fungal mycelium	*S. aureus* ATCC25923 A clinical isolate of MRSA SK1, *E. coli* ATCC25922, *P. aeruginosa* ATCC27853	Microdilution method according to a modification of Clinical and Laboratory Standards Institute (CLSI) M7-A4	Martinez-Klimova et al. (2017)
Xanthomonas oryzae	Oryzae	Rice field	Lauric acid, tridecanoic acid, myristic acid	*Bacillus* strain D13	diffusion method	Muniyan and Gurunathan (2016); Chandra and Singh (2016)

15 endophytic fungi with antibacterial activity	*Dracaena cambodiana* (Asparagaceae); leaf, root and stem *Aquilaria sinensis* (Lour.) Spreng. (Thymelaeaceae); leaf, root and stem	Jinghong city, Xishuangbanna prefecture, Yunnan, China	Crude extract	*B. subtilis* As 1.308, *E. coli* As 1.355, *S. aureus* As 1.72.	Agar diffusion test	Bezerra et al. (2015)
13 isolates of *Phomopsis* sp.	*Aspidosperma tomentosum* MART. (Apocynaceae); leaf Spondias mombin L. (Anacardiaceae); twig	Rio de Janeiro city (Morro do Entorno, Pedra do Marinheiro) and the Brazilian Amazon forest near Redenc, ao (Para state)	Ethyl acetate crude extracts of cultivation broth	*E. coli* (ATCC 25922), *P.aeruginosa* (ATCC 27853), *S. aureus* (ATCC 25,923)	Bioautographic TLC agar-overlay assay	Dokuparthi and Manikanta (2015)
Phomopsis sp. (internal strain no. 8966)	*Notobasis syriaca* (Asteraceae)	ns	Phomosine K, Phomosine A, phenylalanine amide, 2-hydroxymethyl-4,5,6 trihydroxycyclohex-2-en	*E. coli*, *B. megaterium*	Agar diffusion assays; Microdilution method in a 96-well microplate	Radić and Štrukelj (2012)
Phomopsis sp.	*Cistus salviifolius* (internal strain 7852) (Cistaceae)	ns	Pyrenocines J-M	*E. coli*, *B. megaterium*	Agar diffusion method	Radić and Štrukelj (2012)

(continued)

Table 9.4 (continued)

Endophytic fungal strain	Host plant(s) (family), plant part or tissue	Habitat of the host plant	Crude extract/isolated metabolite	Test bacteria	Type of test	Reference
Microsphaeropsis sp. strain 7177	*Zygophyllum fortanesii* (Zygophyllaceae); ns	Gomera, Spain	Fusidienol A 8-Hydroxy-6-methyl-9-oxo-9H-xanthene-1-carboxylic acid methyl ester	*E. coli*	Agar diffusion assay	Abreu et al. (2012)
24 endophytic fungi; *Microdiplodia hawaiiensis* CZ315 (most active isolated strain RGM-02)	*Garcinia mangostana* L. (Clusiaceae); leaf and small branch	Bogor Botanical Gardens, Indonesia	Crude ethyl acetate extracts	*S. aureus* (ATCC 25923), *B. subtilis* (ATCC 6633), *E. coli* (ATCC 25922), *P. aeruginosa* (ATCC 27,853), *S. typhi* (ATCC 14028), *M. luteus* (ATCC 10240)	Disc diffusion method; twofold microtiter broth dilution method	Radji et al. (2011)
Fusarium sp., *Phoma* sp., *Epicoccum nigrum*	*Dendrobium devonianum* Paxton (Orchidaceae); stem and root *Dendrobium thyrsiflorum* (Orchidaceae); stem and root	Longling, Vietnam	Crude ethanol extract of fermentation broth	*E. coli*, *B. subtilis*, *S. aureus*	Agar diffusion method	Xing et al. (2011)
14 species, mainly *Colletotrichum gloeosporioides*, *Alternaria alternata, Guignardia bidwelli*, *Phomopsis archeri* and *Drechslera dematioidea*	*Lippia sidoides* Cham. (Verbenaceae); Leaves and stems	Experimental Station of the Agropecuary Research Company of Pernambuco in Carpina-PE, Brazil	Fungal mycelium	*S. aureus* (ATCC-6538), *B. subtilis* (UFPEDA-16), *E. coli* (ATCC-25922).	Antimicrobial assay using a solid medium (Ichikawa 1971)	De Siqueira et al. (2011)

Colletotrichum gloeosporioides	*Vitex negundo* L. (Lamiaceae); leaf	Botanical Garden, Virudhunagar, Tamil Nadu, India	Hexane crude extract Ethyl acetate crude Methanol crude extract	*S. aureus* (MTCC 3160), *B. subtilis* (MTCC 619), *E. coli* (MTCC 4296), *P. aeruginosa* (MTCC 2488), 10 clinical strains of *S. aureus* obtained from Bose Clinical Laboratory and X-ray (India)	Kirby–Bauer disk diffusion test; Paper disk diffusion method; Broth microdilution method in a 96-well microplate	Arivudainambi et al. (2011)
Pichia guilliermondii Ppf9	*Paris polyphylla* var. *yunnanensis* (Franch) Hand.-Mazz. (Trilliaceae); rhizome	Kunming, China	Helvolic acid	*E. coli* (ATCC 29425), *B. subtilis* (ATCC 11,562), *S. aureus* (ATCC 6538), *S. haemolyticus* (ATCC 29970)	A modified microdilution-colorimetric assay, using the chromogenic MTT reagent	Zhao et al. (2010)
Alternaria sp. strain JCM9.2	*Sonneratia alba* J.E. Smith (Sonneratiaceae); leaf	Dong Zhai Gang Mangrove Garden on Hainan Island, China	Xanalteric acid I Xanalteric acid II Altenusin	*E. coli*, *E. faecium*, *Enterococcus cloacae*, *S. aureus*, *S. pneumonia*, *P. aeruginosa*, *K. pneumonia*	Dilution assay	Kjer et al. (2009)
Chloridium sp. (J.F.H. Beyma) W. Gams & Holubova-Jchova	*Azadirachta indica* A. Juss. (Meliaceae); root	Varanasi district, India	Javanicin	*E. coli*, *Bacillus* sp., *P. aeruginosa*, *P. fluorescens*	Microdilution method in a 96-well microplate	Kharwar et al. (2009)

(continued)

Table 9.4 (continued)

Endophytic fungal strain	Host plant(s) (family), plant part or tissue	Habitat of the host plant	Crude extract/isolated metabolite	Test bacteria	Type of test	Reference
Xylaria sp. YX-28	*Ginko biloba* L. (Ginkgoaceae); twig	Jiangsu and Shandong Provinces, China	7-Amino-4-methylcoumarin	*S. aureus*, *E. coli*, *S. typhi*, *S. typhimurium*, *S. enteritidis*, *Aeromonas hydrophila*, *Yersinia* sp., *V. anguillarum*, *Shigella* sp., *V. parahaemolyticus*	Twofold serial dilutions method	Liu et al. (2008)
Fusarium equiseti, *Guignardia vaccinii*	*Garcinia mangostana* L. (Clusiaceae); leaf, stem, root, fruit, and flower *Garcinia parvifolia* (Miq.) Miq. (Clusiaceae); leaf, stem, root, fruit, and flower	Sungai Rengit Village, Johor, Malaysia	Filtered broth suspension	*S. aureus*, *Listeria monocytogenes*, *B. subtilis*, *Aeromonas hydrophila*, *S. faecalis*	Well diffusion assay	Rukachaisirikul et al. (2008)
Guignardia sp. IFB-E028	*Hopea hainanensis* Merrill & Chun (Dipterocarpaceae)	Hainan Island, China	Monomethylsulochrin Rhizoctonic acid Guignasulfide	*H. pylori*	Agar diffusion method	Mégraud and Lehours (2007)

Aspergillus sp. strain CY725	*Cynodon dactylon* (L.) Pers. (Poaceae); leaf	Sheyang Port on the Yellow Sea	Helvolic acid Monomethylsulochrin Ergosterol 3_-Hydroxy-5_,8_-epidioxy-ergosta-6,22-diene	*H. pylori* (ATCC 43,504), Five clinical isolates obtained from antral biopsies of child and adult patients, *B. subtilis, P. fluorescens, E. coli, S. lutea, S. aureus*	Disk diffusion method	Li et al. (2005)
Rhizoctonia sp. strain Cy064	*Cynodon dactylon* (L.) Pers. (Poaceae); leaf	Jiangsu Province, China	Rhizoctonic acid Monomethylsulochrin Ergosterol 3-,5-,6-Trihydroxyergosta-7,22-diene	*H. pylori* (ATCC 43,504), Five randomly selected clinical strains from antral biopsies from children and adults	Agar dilution method	Ma et al. (2004)
Phoma sp. NG-25	*Saurauia scaberrinae* (Actinidiaceae); stem	Central highlands of Papua New Guinea	Phomodione Usnic acid Cercosporamide	*S. aureus* (ATCC 25,923), *E. coli* (Life Technology 18,290–015)	Disk diffusion assay	Yılmaz et al. (2004)
Colletotrichum sp.	*Artemisia annua* L. (Asteraceae); stem	ns	6-Isoprenylindole-3-carboxylic acid 3b,5a-Dihydroxy-6bacetoxy- ergosta-7,22-diene 3b,5a-Dihydroxy-6bphenylacetyloxye rgosta-7,22-diene	*B. subtilis*, *S. aureus*, *Sarcina lutea*, *Pseudomonas* sp.	Paper-disk assay on LB	Lu et al. (2000)

ns not specified, *MTT* 3-(4,5-dimethylthiazol-2-yl)-2,5-dephenyl tetrazolium bromide

cultures, epigenetic remodeling of fungal secondary metabolites can be achieved (Deepika et al. 2016). In *Cladosporium cladosporioides*, the production of new biomolecules enhanced chemical diversity, with the advantage that this technique does not require strain-dependent genetic manipulation and can be applied to any fungal strain (De la Rosa-García et al. 2018; Spina et al. 2018). Because of the complexity of microbial extracts, advanced analytical methods such as mass spectrometry and metabolomics are fundamental to detect and identify coculture-induced metabolites (Dettmer et al. 2007).

The nanospray desorption electrospray ionisation (n-DESI) combination and imaging mass spectrometry (IMS) have led to the monitoring of metabolite production from live microbial colonies within bacterial communities, thus identifying mass spectral molecular networking when different species coexist (Stasulli and Shank 2016). With a peptidogenomic approach the combination of IMS provides insight into the inter-kingdom interaction between *Pseudomonas aeruginosa* and *Aspergillus fumigatus* at a molecular level, thus allowing the visualisation and identification of metabolites secreted by these microorganisms as grown on agar (Moree et al. 2012).

9.7 Conclusion and Future Prospects

Fungal communities are very diverse and abundant in the environment, and thus they are a versatile reservoir of metabolites with new structures and new bioactivities that can be of potential use as leading compounds to manufacture new modern medicines. Sample collection and fungal cultivation methods in other environments such as terrestrial soil and freshwater and marine areas are very difficult: more fungi have been cultivated from these environments. A potential source for natural bioactive compound or secondary metabolites is provided by these fungal communities rather than a new drug to be extracted. Secondary metabolites extracted from the fungal communities of plant inhabitants with broad bioactivities, such as antifungal, antibacterial, anticancer, antiviral, anti-larval settlement, and cytotoxic activity, have been featured in the literature. In the natural ecosystem these bioactive compounds not only help any environmental fungus to defend against predators, but also have the potential of becoming treatments for human diseases and probes for new biological targets. This chapter indicates that study of the community of fungi characterized by their bioactive metabolites is underway, which is of increased importance as there is an urgent need for new drugs to overcome emerging and drug-resistant diseases.

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Chapter 10
Industrially Important Pigments from Different Groups of Fungi

Ashok Kumar, Srishti Prajapati, Nikhil, Smriti Nandan, and Trisha Guha Neogi

10.1 Introduction

The production of pigments from natural origins has become substantial across the world because of the adverse outcomes of using synthetic colorants. Various plant part materials such as roots, bark, leaves, berries, seeds, twigs, branches, tubers, and nut hulls are capable of producing a wide range of colors with various moderations that can be used for dyeing yarns and in textile industries. Also, when wreathed properly, these natural dyes are fast, efficient, and resist fading from exposure to light. Many companies have decided to utilize these natural pigments from plant and animal sources. The use of these pigment products in the food industry, textile industry, pharmaceuticals, and cosmetics has increased exponentially.

The application of natural dyes is widely used as a colorant agent. Over the decades, several active metabolites have been discovered from distinct natural sources such as microorganisms, insects, animals, and higher plants. Because of their chemo-organotrophic property, the microorganism is the most likely group that generates metabolites possessing the readiest industrial application, and microorganisms also have a high growth rate, producing a large amount of biomass in a short period of time (Dufosse 2006). Many synthetic dyes have been found lethal and dangerous to human health. For reasons of safety, only limited dyestuffs have been acceptable for use in the food industry in many nations. However, as compared to microbial pigments, these additives have several drawbacks such as low water solubility, instability, and unavailability part of the year for industrial applications (Gunasekaran and Poorniammal 2008; Mendez et al. 2011; Yadav et al. 2017).

Pigments also are known as colorants that have water- and oil-insoluble natural as well as synthetic compounds that divulge color to substances such as textiles, paper, or plastics. Pigments change the color of reflected or transmitted light as the

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result of wavelength selective absorption. Because pigments are insoluble in nature, they are applied as finely ground solid particles mixed with a liquid and not as solutions. They are not affected physically or chemically by the substrate in which they are incorporated. A variety of fungi species including *Aspergillus sydowii*, *Aspergillus aureolas*, *Aspergillus keveii*, *Penicillium flavigenum*, *Lecanicillium aphanocladii*, *Penicillium chermesinum*, *Epicoccum nigrum*, and *Fusarium* spp. produce various characteristic pigments (da Costa Souza et al. 2016).

Generally, dark brown or black pigments occur widely in fungi. *Drechslera* spp. produces hydroxyanthraquinones [e.g., helminthosporin (maroon, brown), catenarin (red), cynodontin (bronze), and tritisporin (red-brown)] pigment molecules. In some fungi, both hyphae and conidia are densely pigmented, as in the Dematiaceae family (*Alternaria*, *Curvularia*, *Drechslera*) (Margalith 1992). Health consciousness has prompted the choice of these natural colorants over synthetic colorants in the foods, cosmetics, textiles, and pharma industries. The natural pigments may be present in large quantity but only a few are available in sufficient quantities for industrial production. Production of these natural pigments from microorganisms is advantageous over other methods because microorganisms have a short life cycle and multiply, which results in high productivity (Lauro 1991; Kim et al. 1999).

Natural colorant production from microorganisms has advantages compared with their counterparts extracted from plants or animals because they do not exhibit the problem of seasonal availability and are often more stable and soluble (Gunasekaran and Poorniammal 2008). The synthesis of colorants by plant species is slower than that by microorganisms and algae because the fermentation processes are inherently faster and more productive than other chemical processes (Velmurugan et al. 2010). Food industries from Europe and the US have already obtained natural colorants from microorganisms because of their advantages such as stability, large-scale production, high growth rate, high throughput, and wide range of colors and also their biological activities, such as antimicrobial, antioxidant, and anticancer properties (Pangestuti and Kim 2011; Teixeira et al. 2012; Tuli et al. 2014). The microorganisms produce a diversity of bio-compounds, such as carotenoids, melanins, flavins, quinones, monascines, violaceins, phycocyanins, and indigo (Mapari et al. 2009; Dufossé et al. 2005).

Among the microorganisms, fungi are essential as colorant producers. Filamentous fungi have proved to be useful because of their ability to produce primary and secondary metabolites such as peptides, enzymes, organic acids, heterologous proteins, antibiotics, and pigments (Rana et al. 2018b; Suman et al. 2016; Yadav 2018; Radzio and Kück 1997; Hajjaj et al. 1998). The successful industrial production of β-carotene by *Blakeslea trispora* is the best example, whereas other sources are also present, such as *Mucor circinelloides* (a zygomycete fungus) and *Phycomyces blakesleeanus* (Mapari et al. 2006). Pigments such as cynodontin, helminthosporin, catenarin, chrysophanol, tritisporin, and erythroglaucin are isolated from the fungal species *Eurotium* spp., *Fusarium* spp., *Curvularia lunata*, and *Drechslera* (Rana et al. 2018a; Yadav et al. 2018).

Various strains of *Penicillium oxalicum* produce an extracellular metabolite anthraquinone, which is red. Some non-carotenoid pigments possess a broader

range of color than the limited color range of carotenoid. These pigments are soluble in water and do not require any chemical modification, use of carriers, and stabilizer during dispersion in foods. Extracellular red pigments are isolated in large amounts from *Penicillium marneffei*, and one of these pigments was identified as monascorubramine, the red pigment produced from *Monascus* (Mapari et al. 2005). Microorganisms are noted as being a significant source of naturally occurring pigments. Among all the microorganisms, fungi showed a wide range of fascinating colors. However, until recently, several fungi have remained unexplored for color production, possibly because of their association with aflatoxins, mycotoxins, and other toxic compounds that are harmful for humans.

The increasing urge in society for natural ingredients has compelled biotechnologists to explore novel means and sources for the biotechnological synthesis of food colorants. In this regard, exploring fungal chemical diversity is worthwhile for the identification of novel pigments. The screening approach for water-soluble pigments, which is partially based upon chemotaxonomy, would provide a base for the construction of cell factories to produce natural pigments in the near future. If substantial toxicological testing is carried out, fungal pigments could be accepted for current consumption as a food and textile colorant.

Extracts of *Monascus purpureus* have produced the pigments monascorubrin, rubropunctatin, and citrinin as mycotoxins. The crude filtrates can be used in the textile industry; however, additional pigment purification is required for food and pharmaceutical applications (Lopes et al. 2013). Until now, only a few species were described for pigment production because such pigments were used as a chemotaxonomic tool. The yellow pigments sorbicillin and xanthocillins isolated from the species *Penicillium chrysogenum* were reported (Mapari et al. 2009), and chrysogenin was also reported as a yellow pigment produced by this fungus (Asilonu et al. 2000). *Fusarium graminearum* produces rubrofusarin and aurofusarin, a reddish pigment. This chapter covers different genera of fungi that produce color pigments with various industrial applications using eco-friendly and cost-effective technology.

10.2 Fungal Pigments

Pigments produced by fungi are the secondary metabolite molecules. These molecules are used commercially on a large scale made possible in the same way as antibiotics are mass produced from fungi by deep-tank fermentation. The production of colors from fungal sources is done under controlled experimentation on a mass-scale basis using a wide variety of substrates (Sudha et al. 2014). Using filamentous fungi cultured on different agro-industrial by-products has proved to be an alternative way to obtain pigments (Lopes et al. 2013). Fungi are an essential source of pigment production because some fungi species are abundant in producing stable colorants (Nagia and El-Mohamedy 2007). Some important genera including *Aspergillus*, *Penicillium*, *Epicoccum*, *Lecanicillium*, and *Fusarium* spp. are useful

for producing various colors. *Monascus purpureus*, commonly known as red mold, has been used for food and medicines (Wang and Lin 2007). The pigments of *Penicillium* spp. have been studied extensively, and many yellow-red compounds based on the phenalenone structure have been reported (Bachmann et al. 1986) (Fig. 10.1).

Some fungi produce pigments that belong to the aromatic polyketide groups, such as melanins, quinines (Dufossé et al. 2005; Caro et al. 2012), anthraquinone, flavins, ankaflavin, and naphthoquinone (Dufosse 2006). Fungi have a broad range of biological activities of pharmaceutical properties and are considered to provide a high benefit to humans (Zhang et al. 2004). The use of microbial pigments has benefits including easy and fast growth in inexpensive culture media, different color shades independent of weather conditions, and various industrial applications (Venil and Lakshmanaperumalsamy 2009).

Because of the increasing costs of pollution by raw materials, and the complexity of synthetic material and its products, natural compounds are becoming important. Colorants occurring naturally have antimicrobial properties, are less allergenic, and are very stable, so these are being used instead of synthetic agents (Mehrabian et al. 2000). Moreover, synthetic dyes have environmentally hazardous effects and thus must be replaced by eco-friendly natural dyes (Sewekow 1988; Velmurugan et al. 2010). The ascomycetes fungi species possess an extraordinary color range of pigments in the red and yellow spectra, and these fungal pigments are comparable to existing natural food colorants as a new source for food coloring (Mapari et al. 2006). Many researchers have isolated different genera of fungi to study the production of various pigments (Table 10.1).

During the growth period, fungi such as *Trichoderma*, *Fusarium*, *Penicillium*, and *Aspergillus* produce pigments in the form of intermediate metabolites (Atalla et al. 2011). Secondary metabolites produced by fungal pigments can be classified

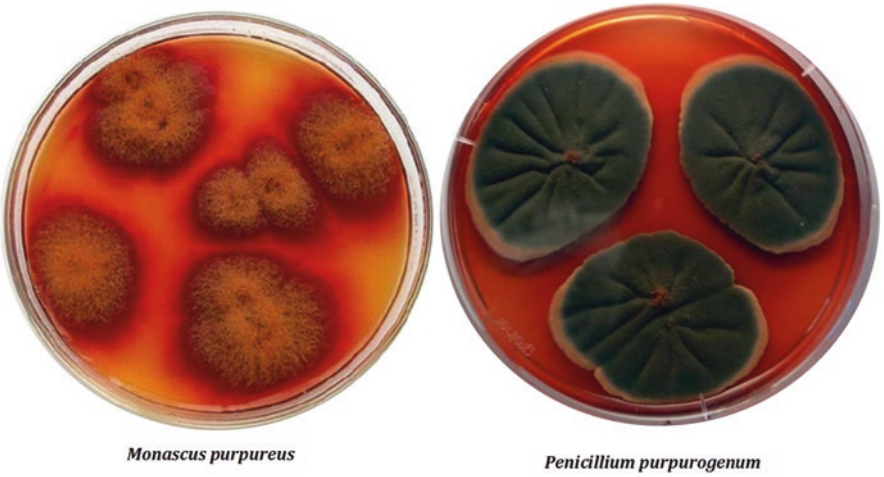

Fig. 10.1 Color of *Monascus purpureus* and *Penicillium purpurogenum* on agar plates

Table 10.1 Fungi producing various types of pigments

Fungi	Pigments	References
Epicoccum nigrum	Carotenoids	Gribanovski-Sassu and Foppen (1967)
Epicoccum nigrum	Flavonoids	Soptica and Bahrim (2005)
Epicoccum nigrum	Polyketides	Shu et al. (1997)
Penicillium chermesinum	Azaphilones	Huang et al. (2011)
Penicillium flavigenum	Anthraquinones and yellow polyketides	Frisvad and Samson (2004)
Fusarium moniliforme	Anthraquinones	Premalatha et al. (2012)
Fusarium verticillioides	Anthraquinones	Boonyapranai et al. (2008)
Lecanicillium aphanocladii (CML2970)	Mycotoxin	Souza et al. (2016)
Epicoccum nigrum (CML2971)	Orevactaene	Souza et al. (2016)
Penicillium flavigenum (CML2965)	Dihydrotichodimerol	Souza et al. (2016)
Talaromyces amestolkiae	Azaphilone	Yilmaz et al. (2012)
Fusarium graminearum	Diacetoxyscirpenol, Fusarenone X and neosolaniol	Nielsen and Smedsgaard (2003)
Monascus purpureus	Monascorubrin and Rubropunctatin	Rasmussen et al. (2011)
Neurospora crassa	Neurosporaxanthin	Aasen and Jensen (1965)
Neurospora sitophila	Neurosporaxanthin	Luque et al. (2012)
Neurospora intermedia (PTCC 5291)	Mixture of carotenoids	Khiabani et al. (2011)
Cordyceps unilateralis	Naphtoquinone	Unagul et al. (2005)
Ashbya gossypi	Riboflavin	Santos et al. (2005)
Monascus spp.	Rubropunctatin	Blanc et al. (1994)
Rhodotorula spp.	Torularhodin	Sakaki et al. (2000)
Blakeslea trispora	β-Carotene	Lampila et al. (1985)
Fusarium sporotrichioides	β-Carotene	Jones et al. (2004)
Auricularia auricula	Melanin	Sun et al. (2016)
Talaromyces verruculosus	Red pigment	Chadni et al. (2017)
Stemphylium lycopersici	Anthraquinone	Li et al. (2017)
Fusarium sp. JN158	Benzoquinone	Zheng et al. (2017)
Monascus anka GIM 3.592	Azaphilone	Chen et al. (2015)
Fusarium fujikuroi	Bikaverin	Lale and Gadre (2016)
Chlorociboria aeruginosa	Quinones	Hinsch et al. (2015)
Grifola frondosa	β-Carotene	Smith et al. (2015)
Fusarium moniliforme	Naphtoquinone	Pradeep and Pradeep (2013)
Monascus purpureus	Azaphilone	Seyedin et al. (2015)

chemically as carotenoids and polyketides, in which polyketides are composed of tetraketides and octoketides with eight C2 units that contribute to the polyketide chain. Anthraquinone, hydroxyanthraquinones, naphthoquinone, and azaphilone represent classes possessing an array in color, and this rational approach is significantly safe for fungal cell factories of polyketide pigments for other industrially important uses(Mapari et al. 2009, 2010).

The class of quinones belonging to the anthraquinone family consists of several hundred compounds that vary in the nature and position of substituent groups (Liu et al. 2008). Anthraquinone has had a significant role in dyestuff industries for a long time. Several fungi including *Trichoderma*, *Aspergillus*, and *Fusarium* are used to isolate anthraquinone compounds through biotechnological techniques, most of which produce a mixture of anthraquinone pigments (Hobson and Wales 1998; Durán et al. 2002). Hydroxyanthraquinone is derived from anthraquinone when one hydrogen atom replaced by a hydroxyl group. Some filamentous fungi species can produce hydroxyanthraquinone compounds by the polyketide pathway for use as food colorants and dyestuffs (Caro et al. 2012).

Napthoquinones are significant in the preparation of dyes, with a full color range between orange, yellow, and brown. The fungal pigment naphthoquinones have a wide range of biological activities and occur extensively in species of *Fusarium* (Mendentsev and Akimenko 1998; Babula et al. 2009). Azaphilones, another group of fungal metabolites that is produced by the genus *Monascus*, have similar molecular structures as well as similar chemical properties (Dufosse 2009). *Monascus* fungi are well known to produce six primary pigments of polyketide origin, classified into three groups based on color: yellow, monascin and ankaflavin; orange, monascorubrin and rubropunctatin; and red, monascorubramine and rubropuntamine (Sweeny et al. 1981) (Fig. 10.2). *Monascus* pigments are sensitive to heat, fade with exposure to light, are not stable at pH ranging from 2 to 10, and have low water solubility. Their stability is affected by various factors including acidity, temperature, light, oxygen, water activity, and time. When these pigments react with amino group-containing compounds in the medium such as protein, amino acids, and nucleic acids, they convert into water-soluble pigments (Dufosse 2009). The fungal species *Epicoccum nigrum* has been identified showing the production of various secondary metabolites including pigments such as carotenoids (Gribanovski-Sassu and Foppen 1967), flavonoids (Soptica and Bahrim 2005), and polyketides (Shu et al. 1997), of red, orange, and yellow hues.

Consequently, *Epicoccum nigrum* should be considered as a potential source of pigments (Mapari et al. 2008). *Penicillium chermesinum* (ZH4-E2), isolated from mangroves, is reported as a producer of new azaphilones (chermesinones). *Penicillium flavigenum* belongs to the section *chrysogena*, known to have strains capable of producing anthraquinones and other yellow polyketides (Frisvad and Samson 2004). Moreover, the two species *P. chrysogenum* and *P. flavigenum* are reported to produce the antibiotic xanthocillin (Frisvad and Samson 2004). Some species of *Aspergillus*, such as *A. glaucus*, *A. cristatus*, and *A. repens*, have been reported as possible sources of pigments.

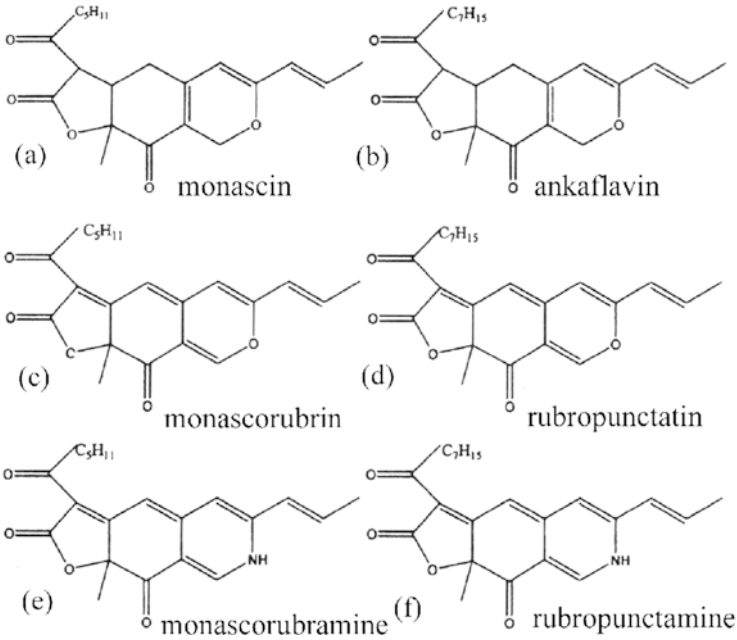

Fig. 10.2 *Monascus* spp. produces six major pigments with different colors

Yellow and red hydroxyanthraquinoid pigments such as emodin and physicion (yellow), questin and erythroglaucin (yellow to orange-brown), and catenarin and rubrocistin (red) are reported (Caro et al. 2012). Extract of *Lecanicillium aphanocladii* (CML2970) produced the pigment oosporein (2,5-dihydroxybenzoquinone), which has mycotoxin properties when extracted initially from the basidiomycete *Oospora colorans* (Kogl and Van Wessem 1944). Oosporein has also been found in other fungi such as *Chaetomium cupreum*, *Verticillium palliate*, *Beauveria* spp., and *Chaetomium trilaterale* (Luo et al. 2015; Mao et al. 2010; Nagaoka et al. 2004). Apart from that, this compound has significant biological activities including inhibition of growth in plants and phytotoxic effects (Cole et al. 1974). Also, oosporein has shown antifungal activity against *Phytophthora infestans* (Nagaoka et al. 2004). *Pythium ultimum*, *Botrytis cinerea*, and *Rhizoctonia solani* have the potential to inhibit the proliferation of tumor cell lines (Mao et al. 2010).

10.3 Industrial Application of Fungal Pigment

The increasing urge in society for natural ingredients has compelled biotechnologists to explore novel means and sources for the synthesis of food colorants. For antioxidant and antimicrobial products, the food industry is facing a severe

challenge, and these products are considered as beneficiary to human health, which reduces the consumption of synthetic chemical preservatives (Vendruscolo et al. 2013). A variety of fungi species obtained from soil niches produces natural colorants with various applications in industry. These colorants are used as additives, color intensifiers, and antioxidants in the food industry and as textile dyes in the textile industry. Moreover, anthraquinones are also used in manufacturing cloth that contains antimicrobial properties.

Pigments have a wide range of colors and some are water soluble. These properties are responsible for the production, isolation, and characterization of many compounds (Durán et al. 2002). At present, the role and use of these pigments are increasing dramatically. It would be hard to find any industry wherein the use of these pigments does not play any significant role. To discover those pigments that have the caliber for long-lasting utilization as well as being environmentally safe is a big challenge for the food industry. Artists' colors are pigments that are spread on a surface suspended in a suitable medium, such as oil. When the pigments exist in the form of dispersions, this could result in the formation of mass coloration for textile fibers, polymers, and rubber.

Monascus was first discovered and used as a natural food colorant in Chinese medicine in the Asian region, although the first classification of *Monascus* strains was performed in other countries (Hamano and Kilikian 2006; Srianta et al. 2014). *Monascus purpureus* produces a red pigment that shows antimicrobial activity whereas the extract of *M. purpureus* was found to be 81% as effective compared with the antibiotic ciprofloxacin (Kumar et al. 2012). AUMC 5705, a strain of *Monascus*, manifested a high production of butyric acid, pyran, and fatty acids having anticancer activity, whereas AUMC 4066 has a significant role in the food, pharmaceutical, and other industries (Moharram et al. 2012). The presence of mycotoxins, for example, citrinin, in some species, has certainly limited the utilization of *Monascus* in food by safety concerns. Meanwhile, during the past 20 years, researchers have demonstrated several molecular pathways and have been trying to inhibit the effect and production of citrinin, thus developing strains incapable of coproducing the citrinin (Wang et al. 2004; Pisareva et al. 2005; Xu et al. 2009).

The biosynthesis of polyketides in several fungi has not been studied in detail at a genetic level. There is still controversy, and the reasons behind those genes responsible for pigment production in *Monascus* are still unclear. However, only a few genes have been reported, such as the MpigE gene, in *Monascus* when it was analyzed for pigment biosynthesis. The complementation, disruption, and overexpression of the MpigE gene had specific effects on pigment biosynthesis, whereas in a fermentation medium the citrinin effects fall exponentially with overexpression of MpigE (Liu et al. 2014).

Monascus spp. have been used as food and medicine for more than 1000 years (Wang and Lin 2007). In China, these fungi have been used for centuries to enhance the color and flavor of foods and have also been used medicinally for several diseases related to vascular and digestive health. Red yeast rice, which has cholesterol-lowering properties, is broadly used as a food supplement in Western countries. Red yeast rice attains the property of lowering cholesterol from the inhibitor monacolin

K. In the fungus *Monascus*, ankaflavine and monascine are yellow pigments, rubropunctatine and monascorubrine are orange, monascorubramin is red, and rubropunctamine and monascorubramine are purple pigments (Blanc et al. 1994).

Sclerotiorin, an aldose reductase inhibitor having a secondary metabolite isolated from *Penicillium sclerotiorum*, is used in several pharmaceutical applications. Large-scale production in liquid culture of *Penicillium sclerotiorum* isolated from Serrado Cipo National Park soil led to the isolation of pencolide, sclerotiorin, and isochromophilone. Some of these compounds, such as pencolide and sclerotiorin, demonstrated antimicrobial activity against gram-positive bacteria (*Salmonella typhimurium, Streptococcus pyogenes, Staphylococcus aureus*), gram-negative bacteria (*Escherichia coli*), and yeast (*Candida albicans*). Antibacterial activity against *S. aureus* is shown by isochromophilone (Lucas et al. 2007). Table 10.2 lists fungi that produce various colors, with their pigments, molecular formula, and applications.

Pigments have all-around features to give credence to their usefulness in a variety of mediums. Some pigments, such as carotenoids and betanins, contain liable hydrogen that shows decolorization as the result of oxidation, which results in their insensitivity to light, heat, and oxygen. During storage and processing, such properties can reduce the robustness of color additives (Mapari et al. 2005). These pigments are produced mainly in the cell-bounded state, although some methods have been patented to make water-soluble pigments. In monascorubrine or rubropunctatine, the replaceable oxygen is substituted by the nitrogen of the amino group of various compounds such as amino acids, peptides, and proteins, with the color change from orange to purple as the basic principle.

Pigment stability is affected by acidity, temperature, light, oxygen, water activity, and time. With the addition of these pigments, sausages or canned pate remained stable for 3 months of storage at 4 °C, and their stability ranged from 92% C to 98% C. (Fabre et al. 1993). Compounds such as anthraquinone, isolated from *Fusarium oxysporum*, are used as natural dyes in dying wool (Nagia and El-Mohamedy 2007). Moreover, the refined and purified form of pigment isolated from *Penicillium purpurogenum* can be used as a natural dye for cotton fabrics and also has antimicrobial activity with good scope for future industries (Velmurugan et al. 2009). These pigments are also used for medicinal purposes in textiles because of the antibacterial properties (Poorniammal et al. 2013). Fungi are the most ideal and versatile model organisms for research on industrial fermentation as well as natural phenomena (Schuster and Schmoll 2012).

10.4 Conclusion and Future Prospects

The indiscriminate use of synthetic dyes for coloration has created harmful effects on living organisms, including human beings, and also caused environmental pollution. Thus, there is a crucial need to identify natural pigment-producing sources for safe colorants. Fungi may have potential in the production of pigments to be used

Table 10.2 Natural occurrence of fungal pigments from soil and their suggested applications

Fungi	Colors	Pigments	Molecular formula	Applications	References
Fusarium oxysporum	Pink/violet	Anthraquinone	$C_{14}H_8O_2$	Textile dyeing Antibacterial activity	Gessler et al. (2013)
Aspergillus niger	Black	Aspergillin	$C_{24}H_{35}NO_4$	Antimicrobial activity	Ray and Eakin (1975)
Aspergillus versicolor	Yellow	Asperversin	$C_{47}H_{58}O_{10}$	Antifungal activity	Miao et al. (2012)
Blakeslea trispora	Cream red	β-Carotene	$C_{40}H_{56}$	Coloring agent use in food and textile industry	European commission (2000)
Candida famata	Yellow	Riboflavin	$C_{17}H_{20}N_4O_6$	Baby foods, breakfast cereals, fruit drinks	Stahmann et al. (2000)
Eurotium spp.	Yellow	Isoquinoline	C_9H_7N	Antifungal activity	Torres et al. (2016)
Fusarium verticillioides	Yellow	Naphthoquinone	$C_{10}H_6O_2$	Dyeing, antibacterial activity	Boonyapranai et al. (2008)
Fusarium sporotrichioides	Red	Lycopene	$C_{40}H_{56}$	Coloring agent	Velmurugan et al. (2009)
Monascus spp.	Yellow	Monascin	$C_{21}H_{26}O_5$	Food colorant Pharmaceuticals	Mostafa and Abbady (2014)
Monascus spp.	Orange	Monascorubrin	$C_{23}H_{26}O_5$	Antibacterial Anticancer activity	Moharram et al. (2012)
Neurospora intermedia	Yellow-orange	β-Carotene	$C_{40}H_{56}$	Various industrial and pharmaceuticals applications	Singgih et al. (2005)
Neurospora sitophila	Orange	Neurosporaxanthin	$C_5H_4N_4O_2$	Antioxidants	Díaz-Sánchez et al. (2011)
Penicillium purpurogenum	Yellow to orange	Mitorubrin	$C_{21}H_{18}O_7$	Antibacterial	Martinkova et al. (1995)
Cordyceps unilateralis	Deep blood-red	Naphtoquinone	$C_{10}H_6O_2$	Activity on various biological oxidative process	Unagul et al. (2005)
Penicillium flavigenum	Yellow	Anthraquinones	$C_{14}H_8O_2$	Antioxidants	Frisvad and Samson (2004)
Penicillium herquei	Yellow	Atronenetin	–	Food additive Antioxidant	Takahashi and Carvalho (2010)

Aspergillus sclerotiorum	Yellow	Neoaspergillic acid	$C_{12}H_{20}N_2O_2$	Antibacterial activity	Micetich and Macdonald (1965)
Penicillium oxalicum	Red	Anthraquinone	$C_{14}H_8O_2$	Anticancer effect in food and pharmaceuticals Textile dyeing	Dufosse (2006)
Penicillium sclerotiorum	Yellow to orange	Pencolide	$C_9H_9NO_4$	Antibacterial activity	Brikinshaw et al. (1963)
Taloromyces astroroseus	Yellow-red	Azaphilone	$C_{21}H_{22}O_7$	Food colorants, cosmetics	Frisvad et al. (2013)
Epicoccum nigrum (CML2971	Orange	Orevactaene	C_{34} $H_{44}O_{10}$	Antioxidant	Souza et al. (2016)
E. nigrum	Red	Carotenoid	$C_{15}H_{10}O_2$	Antioxidants, food coloration Inhibits HIV-1 replication	Gribanovski-Sassu and Foppen (1967)
Trichoderma viride	Yellow Green	Viridin	$C_{20}H_{16}O_6$	Textile dyeing Antifungal activity Food industry	Chitale et al. (2012)
Trichoderma virens	Yellow	Viridol Virone	$C_{20}H_{18}O_6$ $C_{22}H_{24}O_4$	Textile dyeing Antifungal	Mukherjee and Kenerley (2010)
Lecanicillium aphanocladii	Red	Oosporein	$C_{14}H_{10}O_8$	Antifungal activity	Zare and Gams (2001)
Auricularia auricular	Dark tones	Melanin	$C_{18}H_{10}N_2O_4$	Foods, cosmetics, medicine	Sun et al. (2016)
Monascus purpureus	Yellow, orange	β-Carotene	$C_{40}H_{56}$	Antioxidant, medicine	Smith et al. (2015)
Chlorociboria aeruginosa	Green	Quinones	$C_6H_4O_2$	Textile	Weber et al. (2014)
Scytalidium ganodermophthorum	Yellow	Quinones	$C_6H_4O_2$	Textile	Weber et al. (2014)
Monascus ruber	Red	Azaphilone	$C_{21}H_{22}O_7$	Foods	Vendruscolo et al. (2013)
Curvularia lunata	Brown	Anthraquinones	$C_{14}H_8O_2$	Textile	Sharma et al. (2012)
Aspergillus versicolor	–	Asperversin	$C_{24}H_{32}O_8$	Antifungal agent	Miao et al. (2012)

for industrial and biotechnological applications. Production of fungal pigments provides natural coloration without creating harmful effects on entering the environment, a safer alternative use to synthetic colorants. Different genera of fungi produce a variety of pigments including carotenoids, flavonoids, polyketides, azaphilones, anthraquinones, mycotoxin, and orevactaene for important uses in antimicrobials, food colorants, textile dyeing, anticancer activities, food additives, antioxidants, and pharmaceuticals. The natural dyes are easily degradable and also cause no detrimental environmental effects. With the help of biotechnological tools, current advances in the genomic knowledge of fungal species can lead to developing new antifungal drug targets and desired pigments of pharmaceutical importance. The design and development of new antifungal compounds include such benefits for human health as polyketides and statins with fewer side effects. Generally, the critical use of soil filamentous fungi provides an industrially important source of biomass and various valuable products such as pigments, enzymes, and organic acids. Several studies have focused on factors that stimulate pigment production in filamentous and soil fungi. However, these studies have considered optimization of pigment production at a larger scale and potential regulation of different colors of dyes. Thus, substrate, bioreactor design, and cultivation conditions need to be developed and also optimized to control the process for pigments and other desired products.

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Chapter 11
Bioactive Compounds of Endophytic Fungi Associated with Medicinal Plants

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11.1 Introduction

Endophytic fungi are a diverse group of microorganisms that live asymptomatically in different tissues of living plants (Jia et al. 2016). Despite being important components of plant microhabitat (Jia et al. 2016), endophytic fungi are increasingly present in drug discovery programs mainly due to their capability to produce a diversity of secondary metabolites with pharmacological properties. These fungi may help the host plant in defense against attacking microorganisms, predators, and pests and in return receive their nutrition (Strobel and Daisy 2003; Kaul et al. 2012). From the pharmacological applications perspective, endophytic fungi were reported to produce novel antibacterial, antifungal, antiviral, anti-inflammatory, antitumor, antimalarial, and other bioactive compounds (Nisa et al. 2015; Suman et al. 2016).

According to Strobel and Daisy (2003), Strobel et al. (2004), and Yu et al. (2010), several reasonable plant selection strategies should be followed:

1. Plants growing in areas of great biodiversity also have the prospect of housing larger diversity of endophytes.

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2. Plants growing in special habitats, especially those in deteriorated ecological environment, and possessing special capabilities for survival should also be selected for study. People may learn that the power of plants living in such environment may result from the presence of endophytes.
3. Plants surrounded by pathogen-infected plants but showing no symptoms are more likely to lodge endophytes possessing antimicrobial natural products than other plants.
4. Plants that have been exploited for human use as traditional medicines in some place should be considered for study.
5. Plants which occupied a certain ancient land mass are also more likely to lodge endophytes with active natural products than other plants.

The World Health Organization (WHO) defines medicinal plants as "any plant which in one or more of its organs contains substances that can be used for therapeutic purposes or which are precursors for chemo pharmaceutical semi synthesis." They are frequently selected for screening bioactive compounds (Kaul et al. 2012). The research on endophytic fungi increased considerably after the discovery of taxol, one of the most anticancer agents used in the clinic. This diterpenoid can be produced by the endophytic fungi *Taxomyces andreanae* (Strobel 2003), and, from their host, the medicinal plant *Taxus brevifolia* (Stierle et al. 1995). Therefore, from this discovery, it was evidenced that the endophytic fungi might produce the same metabolites of their host plant. However, it is important to highlight that endophytic fungi are also producers of bioactive secondary metabolites that are different from those produced by their hosts and can be of interest for medicinal applications.

11.2 Antibacterial Compounds

Radic and Strukelj (2012) comment on WHO's constant battle against the ever-increasing multidrug resistance of human pathogenic bacteria, highlighting the urgent need for new alternatives to the currently available broad-spectrum antibiotics. According to Boucher et al. (2009), antibiotic resistance has increased in Gram-positive and Gram-negative pathogens, which represent a serious threat to treatment of infectious diseases. Boucher et al. (2009) also highlight the decrease in the development of new antibacterial drugs and reported a decrease of 75% in new antibacterial drugs over the past 25 years that has been approved by the US Food and Drug Administration (FDA).

The secondary metabolites produced by endophytes associated with medicinal plants may have great potential to treat various infectious diseases. These antimicrobial metabolites are low-molecular-weight organic natural substances active at low concentrations against microorganisms (Guo et al. 2000). The first step toward the discovery of new antibacterial compounds produced by endophytic fungi involves the detection of antibiotic activity in fungal culture extracts. However, in some cases, single compounds present in the crude extract do not show significant

antibacterial activity by themselves but can act synergistically in the extract. The identification and structure elucidation of the active metabolite is essential for the development of new antibiotics (Radic and Strukelj 2012). The secondary metabolites with antibacterial activity, isolated from endophytes of medicinal plants between 2008 and 2018, are listed in the Table 11.1.

Liu et al. (2008) suggest that *Xylaria* sp. YX-28, an endophytic fungus isolated from the medicinal plant *Ginkgo biloba* L., discloses a potent antimicrobial activity and could be a valuable source of new antimicrobial drugs. From *Xylaria* sp. YX-28 fermentation broth 7-amino-4-methylcoumarin **(4)** showed strong antibacterial activities in vitro against *Staphylococcus aureus*, *Escherichia coli*, *Salmonella typhi*, *Salmonella typhimurium*, *Salmonella enteritidis*, *Aeromonas hydrophila*, *Yersinia* sp., *Vibrio anguillarum*, *Shigella* sp., and *Vibrio parahaemolyticus* with values of minimal inhibitory concentrations (MIC) ranging from 36 to 142.6 μM. Wu et al. (2018) also studied the endophytic fungi associated with *Ginkgo biloba* L. and obtained *Penicillium cataractum* SYPF 7131, which generated an extract with strong antibacterial activity. From the crude extract of *P. cataractum* SYPF 7131 was isolated the compounds penicimenolidyu A (**67**), penicimenolidyu B (**68**), and rasfonin (**69**) that showed antibacterial activity, mainly, toward *S. aureus*.

A broad diversity of endophytic fungi occurs in the rhizome of *Paris polyphylla* var. *yunnanensis*, a medicinal plant used in traditional Chinese medicine. Some studies have explored the biotechnological potential of these fungi in search of new antimicrobials. Among them, Zhao et al. (2010a) report for the first time the antimicrobial metabolites from the endophytic fungus *Pichia guilliermondii* Ppf9, recovered from rhizome of this plant. From the crude extract of *P. guilliermondii* Ppf9 were obtained three steroids and one nordammarane triterpenoid, ergosta-5,7,22-trienol (**14**), 5α,8α-epidioxyergosta-6,22-dien-3β-ol (**15**), and ergosta-7,22-dien-3β,5α,6β-triol (**16**) and helvolic acid (**17**), which showed activity against *Agrobacterium tumefaciens*, *Escherichia coli*, *Pseudomonas lachrymans*, *Ralstonia solanacearum*, *Xanthomonas vesicatoria*, *Bacillus subtilis*, *Staphylococcus aureus*, and *Staphylococcus haemolyticus*. The helvolic acid (**17**) should be the main antimicrobial component in endophytic fungus *P. guilliermondii* Ppf9 and exhibited the strongest antibacterial activity against *A. tumefaciens*, *E. coli*, *P. lachrymans*, *R. solanacearum*, *X. vesicatoria*, *B. subtilis*, *S. aureus*, and *S. haemolyticus* with MIC values of 2.7, 5.5, 5.5, 2.7, 2.7, 5.5, 87.9, and 10.9 μM, respectively. In addition, from the rhizome of the same plant was obtained the endophytic fungus *Gliomastix murorum* Ppf8, which produced ergosta-5,7,22-trien-3-ol (**33**) and 2,3-dihydro-5-hydroxy-α,α-dimethyl-2-benzofuranmethanol (**34**), compounds that were isolated and shown to be active against *A. tumefaciens*, *E. coli*, *Pseudomonas lachrymans*, *R. solanacearum*, *X. vesicatoria*, *B. subtilis*, and *S. haemolyticus* with the MIC values ranging from 252 to 504 μM. The IC_{50} values of **34** ranged from 140.3 to 366.4 μM (Zhao et al. 2012a). Two sterols and one fatty acid were obtained from the light petroleum extract of the fungus *Fusarium* sp. Ppf4, obtained from the rhizomes of *P. polyphylla* var. *yunnanensis*: 5α, 8α-epidioxyergosta-6, 22-dien-3β-ol (**5**) and ergosta-8(9), 22-diene-3β, 5α, 6β, 7α-tetraol (**6**) and butanedioic acid (**7**). They were assayed against *B. subtilis*, *S. haemolyticus*, *A. tumefaciens*, *E. coli*,

Table 11.1 Antibacterial compounds reported from endophytic fungi recovered from medicinal plants

Fungal endophyte taxa	Medicinal plant/ tissue	Compounds isolated	Biological activity	Minimal inhibition concentration	Reference
Phoma sp.	*Saurauia scaberrinae*/lower crown	**1.** Phomodione ($C_{20}H_{22}O_8$) **2.** Cercosporamide ($C_{16}H_{13}NO_7$) **3.** Usnic acid ($C_{18}H_{16}O_7$)	*Staphylococcus aureus*	2 μg/disk zones of inhibition 0.5 mm	Hoffman et al. (2008)
Xylaria sp.	*Ginkgo biloba*/ twigs	**4.** 7-Amino-4-methylcoumarin ($C_{10}H_9NO_2$)	*Staphylococcus aureus* *Escherichia coli* *Salmonella typhi* *Salmonella typhimurium* *Salmonella enteritidis* *Aeromonas hydrophila* *Yersinia* sp. *Vibrio anguillarum* *Shigella* sp. *Vibrio parahaemolyticus*	91.3 μM 57 μM 114.1 μM 85.6 μM 48.5 μM 22.8 μM 71.3 μM 142.7 μM 36 μM 71.3 μM	Liu et al. (2008)
Fusarium sp.	*Paris polyphylla* var. *yunnanensis*/ rhizomes	**5.** 5α, 8α-epidioxyergosta-6, 22-dien-3β-ol ($C_{28}H_{44}O_3$) **6.** Ergosta-8(9), 22-diene-3β, 5α, 6β, 7α-tetraol ($C_{28}H_{46}O_4$) **7.** Butanedioic acid ($C_4H_6O_4$)	*Bacillus subtilis* *Staphylococcus haemolyticus* *Agrobacterium tumefaciens* *Escherichia coli* *Pseudomonas lachrymans* *Xanthomonas vesicatoria*	349.6–4.5 mM	Huang et al. (2009)
Chloridium sp.	*Azadirachta indica*/ roots	**8.** Javanicin ($C_{15}H_{14}O_6$)	*Escherichia coli* *Bacillus* sp. *Pseudomonas aeruginosa* *Pseudomonas fluorescens*	137.8 μM 137.8 μM 6.9 μM 6.9 μM	Kharwar et al. (2009)

Alternaria sp.	*Sonneratia alba*/ leaves	**9.** Xanalteric acid I ($C_{20}H_{12}O_7$) **10.** Xanalteric acid II ($C_{20}H_{12}O_7$) **11.** Altenusin ($C_{15}H_{14}O_6$)	*Escherichia coli* *Enterococcus faecium* *Enterococcus cloacae* *Staphylococcus aureus* *Streptococcus pneumonia* *Pseudomonas aeruginosa* *Klebsiella pneumonia*	107.6–686 μM	Kjer et al. (2009)
Trichoderma ovalisporum	*Panax notoginseng*/ roots	**12.** Koninginin A ($C_{16}H_{28}O_4$) **13.** Shikimic acid ($C_7H_{10}O_5$)	*Staphylococcus aureus* *Bacillus cereus* *Micrococcus luteus* *Escherichia coli*	5 μg/disk zones of inhibition between 7 and 11 mm	Dang et al. (2010)
Pichia guilliermondii	*Paris polyphylla* var. *yunnanensis*/ rhizomes	**14.** Ergosta-5,7,22-trienol ($C_{28}H_{44}O$) **15.** 5α,8α-epidioxyergosta-6,22-dien-3β-ol ($C_{28}H_{44}O_3$) **16.** Ergosta-7,22-dien-3β,5α,6β-triol ($C_{28}H_{46}O_3$) **17.** Helvolic acid ($C_{33}H_{44}O_8$)	*Agrobacterium tumefaciens* *Escherichia coli* *Pseudomonas lachrymans* *Ralstonia solanacearum* *Xanthomonas vesicatoria* *Bacillus subtilis* *Staphylococcus aureus* *Staphylococcus haemolyticus*	2.7–88 μM	Zhao et al. (2010a)
Phomopsis sp.	*Cistus monspeliensis*	**18.** Phomochromone A ($C_{12}H_{14}O_3$) **19.** Phomochromone B ($C_{12}H_{14}O_4$) **20.** Phomotenone ($C_{11}H_{18}O_2$) **21.** (1*S*,2*S*,4*S*)-trihydroxy-p-menthane ($C_{10}H_{20}O_3$)	*Escherichia coli* *Bacillus megaterium*	0.05 mg/disk zones of inhibition between 6 and 8 mm	Ahmed et al. (2011)
Unidentified ascomycete	*Arbutus unedo*	**22.** Pestalotheol E ($C_{16}H_{24}O_5$) **23.** Pestalotheol F ($C_{16}H_{24}O_5$) **24.** Pestalotheol G ($C_{16}H_{22}O_6$) **25.** Pestalotheol H ($C_{16}H_{24}O_3$) **26.** Anofinic acid ($C_{12}H_{12}O_3$)	*Escherichia coli* *Bacillus megaterium*	50 mg/disk zones of inhibition between 7 and 12 mm	Qin et al. (2011)

(continued)

Table 11.1 (continued)

Fungal endophyte taxa	Medicinal plant/ tissue	Compounds isolated	Biological activity	Minimal inhibition concentration	Reference
Fusarium solani	*Taxus baccata*/bark	**27.** 1-tetradecene ($C_{14}H_{28}$) **28.** 8-octadecanone ($C_{18}H_{36}O$) **29.** 8-pentadecanone ($C_{15}H_{30}O$) **30.** Octylcyclohexane ($C_{14}H_{28}$) **31.** 10-nonadecanone ($C_{19}H_{38}O$)	*Staphylococcus aureus* *Staphylococcus epidermidis* *Bacillus subtilis* *Klebsiella pneumonia* *Escherichia coli* *Shigella flexneri*	1 μg/disk zones of inhibition between 16.3 and 27 mm	Tayung et al. (2011)
Penicillium chrysogenum	*Porteresia coarctata*/leaves	**32.** (3,10-didehydro-3[2(3,3-dimethyl-prop-2-enyl)-3-indolylmethylene]-6-methyl pipera-zine-2,5-dione) ($C_{19}H_{21}O_2N_3$)	*Vibrio cholerae*	10 μg/disk zones of inhibition between 14 and 16 mm	Devi et al. (2012)
Gliomastix murorum	*Paris polyphylla* var. *yunnanensis*/ rhizomes	**33.** Ergosta-5,7,22-trien-3-ol ($C_{28}H_{44}O$) **34.** 2,3-dihydro-5-hydroxy-α,α-dimethyl-2-benzofuranmethanol ($C_{11}H_{14}O_3$)	*Agrobacterium tumefaciens* *Escherichia coli* *Pseudomonas lachrymans* *Ralstonia solanacearum* *Xanthomonas vesicatoria* *Bacillus subtilis* *Staphylococcus haemolyticus*	252–504 μM	Zhao et al. (2012a)
Aspergillus sp.	*Eucommia ulmoides*/roots	**35.** Ergosterol ($C_{28}H_{44}O$) **36.** Cerevisterol ($C_{28}H_{46}O_3$) **37.** 5-Hydroxymethylfuran-3-carboxylic acid ($C_6H_6O_4$) **38.** 5-Methoxymethylfuran-3-carboxylic acid ($C_7H_8O_4$) **39.** Allantoin ($C_4H_6N_4O_3$) **40.** Trypacidin ($C_{18}H_{16}O_7$) **41.** Monomethylsulochrin ($C_{18}H_{18}O_7$)	*Bacillus subtilis* *Staphylococcus aureus* *Staphylococcus faecalis* *Escherichia coli* *Pseudomonas aeruginosa* *Salmonella typhimurium*	7–993 μM	Zhang et al. (2014a)
Botryosphaeria dothidea	*Melia azedarach*/ steam	**42.** Pycnophorin ($C_{20}H_{33}O$)	*Bacillus subtilis* *Staphylococcus aureus*	86.3 μM	Xiao et al. (2014a)

Diaporthe sp.	*Mahonia fortunei*/ leaves	**43.** 19-norlanosta-5(10),6,8,24-tetraene-1α,3β,12β,22S- tetraol ($C_{29}H_{44}O_4$)	*Staphylococcus aureus* *Escherichia coli* *Bacillus subtilis* *Pseudomonas aeruginosa* *Streptococcus pyogenes*	10.9 μM 10.9 μM 4.4 μM 4.4 μM 219 nM	Li et al. (2015a)
Phomopsis liquidambaris	*Cryptolepis buchanani*/steam	**44.** Oblongolide Y ($C_{17}H_{26}O_3$)	*Escherichia coli* *Bacillus subtilis* *Pseudomonas aeruginosa*	89.8 μM 179.6 μM 359.2 μM	Rao & Satish (2015)
Colletotrichum sp.	*Buxus sinica*/leaves	**45.** Colletotrichone A ($C_{18}H_{20}O_7$) **46.** Colletotrichone B ($C_{18}H_{20}O_5$) **47.** Colletotrichone C ($C_{18}H_{22}O_5$)	*Staphylococcus aureus* *Escherichia coli* *Bacillus subtilis*	287–15.8 μM	Wang et al. (2016a)
Penicillium sp.	*Pinellia ternate*/ tubers	**48.** (2*R*)-3′-methoxyl citreovirone ($C_{13}H_{17}O_4Cl_2$) **49.** Helvolic acid ($C_{33}H_{44}O_8$) **50.** Cis-bis-(methylthio)-silvatin ($C_{20}H_{30}N_2O_5S_2$) **51.** Citreovirone ($C_{12}H_{14}Cl_2O_4$) **52.**Trypacidin A ($C_{18}H_{16}O_7$)	*Pseudomonas aeruginosa* *Staphylococcus aureus* *Bacillus subtilis* *Escherichia coli*	8.1–220.7 μM	Yang et al. (2017)
Fusarium solani	*Chlorophora regia*/ roots	**53.** Compounds 1/2 ($C_{21}H_{27}O_6N$) **54.** Compounds 3/4 ($C_{21}H_{27}O_7N$) **55.** Compound 5 ($C_{21}H_{25}O_8N$) **56.** Compound 6 ($C_{22}H_{29}O_7N$) **57.** Compound 7 ($C_{22}H29O_5N$) **58.** Compound 8/9 ($C_{21}H_{27}O_6N$)	*Staphylococcus aureus* *Escherichia coli* *Bacillus subtilis* *Acinetobacter* sp.	11.9–23.8 μM	Kyekyeku et al. (2017)
Epicoccum nigrum	*Ferula sumbul*/leaves	**59.** Di-(2-ethylhexyl) phthalate ($C_{24}H_{38}O_4$)	*Bacillus subtilis* *Staphylococcus aureus* *Escherichia coli*	8 μM 3.7 μM 14.8 μM	Perveen et al. (2017)

(continued)

Table 11.1 (continued)

Fungal endophyte taxa	Medicinal plant/ tissue	Compounds isolated	Biological activity	Minimal inhibition concentration	Reference
Chaetomium sp.	*Scencio stapeliiformis*/arial part	**60.** p-hydroxybenzaldehyde ($C_7H_6O_2$) **61.** Uracil ($C_4H_4N_2O_2$) **62.** 5 effectively ($C_{11}H_{18}N_2O_2$)	*Escherichia coli* *Staphylococcus aureus*	10 µg/disk zones of inhibition between 9 and 11 mm	Tawfik et al. (2017)
Diaporthe terebinthifolii	*Glycyrrhiza glabra*/rhizomes	**63.** (2*E*,4*E*)-6-hydroxynona-2,4-dienoic acid ($C_9H_{14}O_3$) **64.** (*E*)-6-hydroxynon-2-enoic acid ($C_9H_{16}O_3$) **65.** Xylarolide **66.** Phomolide G ($C_{12}H_{20}O_5$)	*Yersinia enterocolitica*	78.4 µM 73.4 µM 72.1 µM 69.2 µM	Yedukondalu et al. (2017)
Penicillium cataractum	*Ginkgo biloba*/ branch	**67.** Penicimenolidyu A ($C_{12}H_{14}O_6$) **68.** Penicimenolidyu B ($C_{13}H_{16}O_7$) **69.** Rasfonin ($C_{25}H_{38}O_6$)	*Staphylococcus aureus* *Bacillus subtilis* *Pseudomonas aeruginosa* *Klebsiella pneumonia* *Escherichia coli*	23–361 µM	Wu et al. (2018)

P. lachrymans, and *X. vesicatoria*, disclosing MIC values in the range 349.6 μM to 4.47 mM and IC_{50} values from 202 μM to 1.5 mM (Huang et al. 2009).

Li et al. (2015a) analyzed secondary metabolites from the endophytic fungus *Diaporthe* sp. LG23 recovered from leaves of *Mahonia fortunei* (Berberidaceae), a medicinal plant used in China as a potent antimicrobial medicine for treating pneumoconiosis, psoriasis, and cough. From *Diaporthe* sp. LG23, a new lanosterol derivative, 19-norlanosta-5(10),6,8,24-tetraene-1α,3β,12β,22S-tetraol **(43)** and six biosynthetically related known ergosterol derivatives were identified. Compound 19-norlanosta-5(10),6,8,24-tetraene-1α,3β,12β,22S-tetraol **(43)**, an unusual fungus-derived 19-nor-lanostane tetracyclic triterpenoid, exhibited pronounced antibacterial efficacy against both Gram-positive and Gram-negative bacteria, especially against clinical isolates of *Streptococcus pyogenes*, *Pseudomonas aeruginosa*, and *S. aureus*, exhibiting MIC values between 0.2 and 10.9 μM.

Wang et al. (2016a) evaluated the antibacterial potential of *Colletotrichum* sp. BS4 using the OSMAC (One Strain Many Compounds) approach. This fungus was recovered from leaves of the medicinal plant *Buxus sinica*, and after fractionation of its extracts, three new compounds were isolated and identified: colletotrichones A – C (**45–47**). Compound colletotrichone A (**45**) showed pronounced activity against *E. coli* and *B. subtilis*, with MIC values of 0.3 and 2.9 μM, respectively, values comparable to that of standard antibiotics. Additionally, colletotrichone C (**47**) was quite active against the environmental strain of *E. coli*, with MIC value of 15.7 μM. Furthermore, colletotrichone B (**46**) was as active as streptomycin against the clinically relevant RG2 bacterium *S. aureus*, with MIC value of 15.8 μM. Moreover, the authors suggest that *Colletotrichum* sp. BS4 provides some form of azaphilone-mediated chemical defense to the host plant against invading specialist and generalist bacteria.

Perveen et al. (2017) characterized the secondary metabolites of the endophytic fungus *Epicoccum nigrum*, recovered from leaves of medicinal plant *Ferula sumbul*. Compound di-(2-ethylhexyl) phthalate (**69**) was purified, and its antibacterial potential was evaluated against *B. subtilis*, *S. aureus*, and *E. coli*, showing promising activity with MIC values 8, 3.8, and 14.9 μM, respectively.

11.3 Antifungal Compounds

According to Vallabhaneni et al. (2015), fungal diseases are a considerable cause of morbidity and mortality globally. The treatment of mycoses has several limitations, such as undesirable side effects, narrow activity spectrum, and a small number of targets and fungal resistance, all of which corroborates the urgent need to develop new therapeutic strategies (Fuentefria et al. 2018). As for medicine, the agriculture needs novel antifungal compounds against phytopathogenic fungi, which are responsible for great losses in the world agricultural production. The secondary metabolites produced by endophytes associated with medicinal plants may be used

for the fungal treatment. The most important antifungal secondary metabolites from endophytic fungi recovered from medicinal plants, characterized between 2012 and 2018, are listed in Table 11.2 (compounds **1–116**).

Carvalho et al. (2018) reported the antifungal activity of the compounds cytochalasin H (**117**) and J (**118**) isolated from crude extracts of the endophytic fungi *Diaporthe miriciae*, UFMGCB 7719 and UFMGCB 6350, recovered from *Copaifera pubiflora* and *Melocactus ernestii*, respectively, in Brazil. The compounds were tested against the fungal plant pathogens *Colletotrichum fragariae*, *C. gloeosporioides*, *C. acutatum*, *Botrytis cinerea*, *Fusarium oxysporum*, *Phomopsis obscurans*, and *P. viticola* using microdilution broth assays. Cytochalasins H and J showed minor mycelial growth stimulation (hormesis) of *B. cinerea*, *C. acutatum*, *C. fragariae*, *C. gloeosporioides*, and *F. oxysporum*. The cytochalasins at a concentration of at 300 μmol L^{-1} caused, after 144 h, 73% and 36% growth inhibition of *P. obscurans*, respectively, and inhibited the growth of *P. viticola* by 61% and 58%, respectively. Chapla et al. (2014b) also isolated cytochalasin H (**119**) from the endophytic fungi *Phomopsis* sp. obtained from leaves of *Senna spectabilis*. The compound demonstrated antifungal activity against *Cladosporium cladosporioides* and *C. sphaerospermum* inhibiting the fungal growth at 10 and 25 μg/spot, respectively.

Zhang et al. (2014b) reported another cytochalasin from the ethyl acetate extract of the endophyte *Xylaria* sp. XC-16, recovered from leaves of *Toona sinensis*. The bioassay-guided fractionation resulted in the isolation of new cytochalasins Z_{27} (**55**) and Z_{28} (**56**), along with three known compounds *seco*-cytochalasin E (**57**), cytochalasin Z_{18} (**58**), and cytochalasin E (**59**). The anti-phytopathogenic activity of the cytochalsins was evaluated on *Fusarium solani*, *Gibberella saubinetti*, *B. cinerea*, and *Alternaria solani*. Compound **56** showed fungicidal effect against *G. saubinetti* with MIC of 12.5 μM, a value comparable with that of the positive control hymexazol (MIC of 25 μM). In contrast, other compounds displayed MIC values greater than 50 μM against the tested pathogens (Zhang et al. 2014b).

Phomopsis sp. YM 355364, a fungi obtained from *Aconitum carmichaeli* growing in China (Wu et al. 2013a), produces the new steroids (14*β*,22*E*)-9,14-dihydroxyergosta-4,7,22-triene-3,6-dione (**106**) and (5*α*,6*β*,15*β*,22*E*)-6-ethoxy −5,15-dihydroxyergosta-7,22-dien-3-one (**107**), along with those of calvasterols A and B (**108**–**109**) and ganodermaside D (**110**). Compound **106** exhibited antifungal activities against *Candida albicans*, *Hormodendrum compactum*, and *Aspergillus niger*, with MIC values of 145.3, 145.3, and 290.5 μM. Compound **107** showed weak inhibitory activity against *C. albicans* and *Fusarium avenaceum* with MIC of 270.8 μM. Compounds **108** and **110** showed moderate inhibitory activities against *F. avenaceum* at 151.4 and 156.6 μM, respectively. Compound **108** exhibited weak antifungal activities against *Pyricularia oryzae* and *Trichophyton gypseum* with MIC values of 302.9 and 605.8 μM, respectively (Wu et al. 2013a).

Xiao et al. (2013) isolated 80 endophytic fungi from healthy leaves and small branches of *Ginkgo biloba* (China). All the fungi were tested in an antifungal bioassay against *Fusarium graminearum*, *Sclerotinia sclerotiorum*, and *Phytophthora capsici* by the agar diffusion method. Fifteen endophytes were active against at least one of the tested fungi, and *Chaetomium globosum* CDW7 yielded the most

Table 11.2 Antifungal compounds reported from endophytic fungi associated with medicinal plants during 2012–2018

Fungal endophyte taxa	Medicinal Plant/ Tissue	Compounds isolated	Biological activity	EC_{50}/ IC_{50}/MIC	Reference
Fusarium sp.	*Mentha longifolia*/roots	**1.** Fusaripeptide A ($C_{46}H_{75}N_7O_{11}$)	*Candida albicans* *Candida glabrata* *Candida krusei* *Aspergillus fumigates*	0.1 μM 0.2 μM 0.2 μM 0.1 μM	Ibrahim et al. (2018)
Penicillium sp.	*Nerium indicum*/ root	**2**. 3-O-methylviridicatin ($C_{16}H_{13}NO_2$) **3.** Viridicatol ($C_{15}H_{11}NO_3$) **4.** 5-hydroxy-8-methoxy-4-phenylisoquinolin-1(2*H*)-one ($C_{16}H_{13}NO_3$)	*Fusarium graminearum*	**2.** 248.7 μM **3.** 493.6 μM **4.** 467.7 μM	Ma et al. (2017)
			Colletotrichum gloeosporioides	**2.** 497.5 μM **3.** 246.8 μM **4.** 467.7 μM	
			Setosphearia turcica	**2.** 497.5 μM **3.** 246.8 μM **4.** 467.7 μM	
			Alternaria alternata	**2.** 124.2 μM	
			Alternaria brassicae	**3.** 246.8 μM **4.** 116.7 μM **2.** 124.2 μM **3.** 123.2 μM **4.** 116.7 μM	
			Sclerotinia sclerotiorum	**2.** 497.5 μM **3.** 246.8 μM **4.** 467.7 μM	
			Botrytis cinerea	**2.** 497.5 μM **3.** 123.2 μM **4.** 116.7 μM	

(continued)

Table 11.2 (continued)

Fungal endophyte taxa	Medicinal Plant/ Tissue	Compounds isolated	Biological activity	EC_{50}/ IC_{50}/MIC	Reference
			Phytophthora capsici	**2.** 994.9 μM **3.** 246.8 μM **4.** 935.4 μM	
			Valsa mali	**2.** 124.2 μM **3.** 123.2 μM **4.** 233.8 μM	
			Peony anthracnose	**2.** 248.7 μM **3.** 246.8 μM **4.** 233.8 μM	
Emericella sp.	*Panax notoginseng*/leaf	**5.** 5-(undeca-3′,5′,7′-trien-1′-yl)furan-2-ol ($C_{15}H_{20}O_2$) **6.** 5-(undeca-3′,5′,7′-trien-1′-yl) furan-2-carbonate ($C_{16}H_{20}O_4$)	*Rhizoctonia solani*	**5.** 107.6 μM **6.** 361.9 μM	Wu et al. (2017)
			Verticillium dahliae	**5.** 27.1 μM **6.** 90.5 μM	
			Helminthosporium maydis	**5.** 13.3 μM **6.** 45.2 μM	
			Fusarium oxysporum	**5.** 107.6 μM **6.** 361.9 μM	
			Fusarium tricinctum	**5.** 107.6 μM **6.** 180.9 μM	
			Botryosphaeria dothidea	**5.** 53.8 μM **6.** 90.5 μM	
			Alternaria fragariae	**5.** 107.6 μM **6.** 180.9 μM	
Chaetomium globosum	*Ginkgo biloba*/leaf	**7.** Chaetoglobosin A ($C_{32}H_{36}N_2O_5$) **8.** Chaetoglobosin D ($C_{32}H_{36}N_2O_5$)	*Sclerotinia sclerotiorum*	**7.** 0.6 μM **8.** 1.2 μM	Zhao et al. (2017)

Fusarium chlamydosporium	*Anvillea garciniil* leaf	**9.** Fusarithioamide A (2-(2-aminopropanamido)-*N*-(1-hydroxy-3-mercaptopropyl) benzamide) ($C_{13}H_{19}N_3O_3S$)	*Candida albicans*	8.7 μM	Ibrahim et al. (2016)
Fusarium fujikuroi	*Eleusine coracana*/shoots	**10.** 5-hydroxy 2(3H)-benzofuranone ($C_8H_6O_3$)	*Fusarium graminearum*	**10.** 208.2 μM	Mousa et al. (2016)
Penicillium chrysogenum	*Eleusine coracana*/roots	**11.** Dehydrocostus lactone ($C_{15}H_{18}O_2$)	*Fusarium graminearum*	**11.** 1.1 mM	Mousa et al. (2016)
Penicillium expansum	*Eleusine coracana*/roots	**12.** Harpagoside ($C_{24}H_{30}O_{11}$)	*Fusarium graminearum*	**12.** 63.2 μM	Mousa et al. (2016)
Chaetomium globosum	*Panax notoginseng*/ seeds	**13.** Chaetoglobosin A ($C_{32}H_{36}N_2O_5$) **14.** Chaetoglobosin B ($C_{32}H_{36}N_2O_5$) **15.** Chaetoglobosin E ($C_{32}H_{38}N_2O_5$) **16.** Chaetoglobosin F ($C_{32}H_{38}N_2O_5$) **17.** Penochalasin G ($C_{32}H_{38}N_2O_4$) **18.** Chaetomugilin A ($C_{23}H_{27}ClO_7$) **19.** Chaetomugilin D ($C_{23}H_{27}ClO_6$) **20.** Flavipin ($C_9H_8O_5$)	*Phoma herbarum*	**13.** 121.1 μM **14.** 30.3 μM **15.** 120.6 μM **16.** 30.2 μM **17.** 124.4 μM **18.** 283.9 μM **19.** 294.3 μM **20.** 2.6 mM	Li et al. (2016a)
			Epicoccum nigrum	**13.** 30.3 μM **14.** 15.1 μM **15.** 7.5 μM **16.** <1.9 μM **17.** <1.9 μM **18.** 17.7 μM **19.** 36.8 μM **20.** 1.3 mM	

(continued)

Table 11.2 (continued)

Fungal endophyte taxa	Medicinal Plant/ Tissue	Compounds isolated	Biological activity	EC_{50}/ IC_{50}/MIC	Reference
Diaporthe maritima	*Picea rubens*/ needles	**21.** Phomopsolide A ($C_{15}H_{18}O_6$) **22.** Phomopsolide B ($C_{15}H_{20}O_6$) **23.** Phomopsolide C ($C_{15}H_{18}O_6$) **24.** Alpha pyrone ($C_{10}H_{10}O_4$)	*Microbotryum violaceum* *Saccharomyces cerevisiae*	**21.** 25 μM **22.** 250 μM **23.** 250 μM **24.** 250 μM	Tanney et al. (2016)
Pestalotiopsis foedan	*Bruguiera sexangula*/branch	**25.** (3*R*,4*R*,6*R*,7*S*)-7-hydroxyl-3,7-dimethyl-oxabicyclo[3.3.1]nonan-2-one ($C_{10}H_{16}O_3$) **26.** (3*R*, 4*R*)-3-(7-methylcyclohexenyl)-propanoic acid	*Botrytis cinerea*	**25.** 16.8 μM **26.** 3.1 μg ml^{-1}	Xu et al. (2016)
			Phytophthora nicotianae	**25.** 34.2 μM **26.** 6.3 μg ml^{-1}	
			Candida albicans	**25.** >542.8 μM **26.** 50 μg ml^{-1}	
Rhizopycnis vagum	*Nicotiana tabacum*/root	**27.** Rhizopycnin D ($C_{14}H_9ClO_5$) **28.** TMC-264 ($C_{16}H_{13}ClO_7$)	*Magnaporthe oryzae*	**27.** 33.8 μM **28.** 34 μM	Lai et al. (2016)
Fusarium sp.	*Ficus carica*/leaf	**29.** Helvolic acid methyl ester ($C_{34}H_{46}O_8$) **30.** Helvolic acid ($C_{33}H_{44}O_8$) **31.** Hydrohelvolic acid	*Botrytis cinerea*	**29.** 42.9 μM **30.** 44 μM **31.** 25 μg ml^{-1}	Liang et al. (2016)
			Colletotrichum gloeosporioides	**29.** 21.5 μM **30.** 22 μM **31.** 12.5 μg ml^{-1}	
			Fusarium oxysporum	**29.** 42.9 μM **30.** 22 μM **31.** 12.5 μg ml^{-1}	
			Fusarium graminearum	**29.** 21.5 μM **30.** 44 μM **31.** 25 μg ml^{-1}	
			Phytophthora capsici	**29.** 21.5 μM **30.** 22 μM **31.** 12.5 μg ml^{-1}	

Trichoderma sp.	*Myoporum bontioides*/root	**32.** Dichlorodiaportinolide ($C_{16}H_{14}Cl_2O_7$) **33.** Dichlorodiaportin ($C_{13}H_{12}Cl_2O_5$)	*Colletotrichum musae*	**32.** 64.2 μM **33.** 470 μM	Li et al. (2016b)
			Rhizoctonia solani	**32.** 16.1 μM **33.** 470 μM	
Trichoderma koningiopsis	*Panax notoginseng*	**34**. Koninginin O ($C_{16}H_{24}O_4$) **35**. Koninginin Q ($C_{17}H_{28}O_5$) **36**. 7-O-methylkoninginin D ($C_{17}H_{28}O_5$)	*Fusarium oxysporum,*	**34.** 456.6 μM **35.** 409.7 μM **36.** NA	Liu et al. (2016a)
			Plectosphaerella cucumerina	**34.** 456.6 μM **35.** 409.7 μM **36.** 409.7 μM	
Trichoderma koningiopsis	*Panax notoginseng*	**37.** Koningiopisin B ($C_{20}H_{34}O_5$) **38.** Koningiopisin C ($C_{16}H_{24}O_4$) **39.** Koningiopisin H ($C_{16}H_{24}O_4$)	*Alternaria panax*	**37.** 180.5 μM **38.** 228.3 μM **39.** 228.3 μM	Liu et al. (2016b)
			Fusarium oxysporum	**37.** NA **38.** 114.1 μM **39.** NA	
			Plectosphaerella cucumerina	**37.** NA **38.** 57.1 μM **39.** NA	
			Fusarium solani	**37.** NA **38.** 114.1 μM **39.** NA	

(continued)

Table 11.2 (continued)

Fungal endophyte taxa	Medicinal Plant/ Tissue	Compounds isolated	Biological activity	EC_{50}/ IC_{50}/MIC	Reference
Pestalotiopsis sp.	*Dendrobium officinale*/shoots	**40.** (4*S*,6*S*)-6-[(1*S*,2*R*)-1,2-dihydroxypentyl]-4-hydroxy-4-methoxytetrahydro-2*H*-pyran-2-one ($C_{11}H_{20}O_6$) **41.** (6*S*,2*E*)-6-hydroxy-3-methoxy-5-oxodec-2-enoic acid ($C_{11}H_{18}O_5$) **42.** LL-P880γ **43.** LL-P880α **44.** Ergosta-5,7,22-trien-3β-ol	*Candida albicans*	**40.** 25.2 μM **41.** 54.3 μM **42.** 12.5 μg ml^{-1} **43.** 6.3 μg ml^{-1} **44.** >400 μg ml^{-1}	Wu et al. (2016)
			Cryptococcus neoformans	**40.** 12.6 μM **41.** 54.3 μM **42.** 50 μg ml^{-1} **43.** 3.1 μg ml^{-1} **44.** 200 μg ml^{-1}	
			Trichophyton rubrum	**40.** 100.7 μM **41.** 27.1 μM **42.** 50 μg mL^{-1} **43.** 50 μg mL^{-1} **44.** >400 μg mL^{-1}	
			Aspergillus fumigatus	**40.** 100.7 μM **41.** 13.6 μM **42.** 50 μg ml^{-1} **43.** 25 μg ml^{-1} **44.** >400 μg ml^{-1}	
Aspergillus terreus	*Carthamus lanatus*/roots	**45.** (22*E*,24*R*)-stigmasta5,7,22-trien-3-β-ol **46.** (*R*)-methyl 4-ethoxy-2-(4-hydroxy-3-(3-methylbut-2-enyl) benzyl)-3-(4-hydroxyphenyl)-5-oxo-2,5-dihydrofuran-2-carboxylate (aspernolide F) ($C_{26}H_{28}O_7$)	*Cryptococcus neoformans*	**45.** 4.4 μg mL^{-1} **46.** 11.5 μM	Ibrahim et al. (2015)

Mycosphaerella sp.	*Eugenia bimarginata*/leaf	**47.** (2*S*,3*R*,4*R*)-(*E*)-2-amino-3,4-dihydroxy-2-(hydroxymethyl)-14-oxoeicos-6,12-dienoic acid ($C_{21}H_{37}NO_6$) **48**. Myriocin ($C_{21}H_{39}NO_6$)	*Cryptococcus neoformans*	**47.** 3.3 μM **48.** 1.2 μM	Pereira et al. (2015)
			Cryptococcus gattii	**47.** 6.3 μM **48.** 1.24 μM	
Xylaria sp.	*Azadirachta indica*/stems	**49.** Guaiane-2,10,11,12-tetraol ($C_{15}H_{28}O_4$) **50.** Guaiane-2,4,10,11,12-pentaol ($C_{15}H_{28}O_5$) **51.** Guaiane-4,5,10,11,12-pentaol ($C_{15}H_{28}O_5$) **52.** Guaiane-1,5,10,11,12- pentaol ($C_{15}H_{28}O_5$) **53.** 11-methoxyguaiane-4,10,12- triol ($C_{16}H_{30}O_4$)	*Candida albicans*	**49.** 1.9 mM **50.** 443.9 μM **51.** 221.9 μM **52.** 111 μM **53.** 111.7 μM	Huang et al. (2015)
			Aspergillus niger	**49.** 939.9 μM **50.** >1.8 mM **51.** 221.9 μM **52.** 887.7 μM **53.** 893.9 μM	
			Pyricularia oryzae	**49.** 469.9 μM **50.** 887.7 μM **51.** 887.7 μM **52.** 111 μM **53.** 893.9 μM	
			Hormodendrum compactum	**49.** 939.9 μM **50.** 887.7 μM **51.** 221.9 μM **52.** 221.9 μM **53.** 893.9 μM	
Trichoderma brevicompactum	*Allium sativum*	**54.** 4β-acetoxy-12,13- epoxy-Δ^9-trichothecene (trichodermin) ($C_{17}H_{24}O_4$)	*Rhizoctonia solani* *Botrytis cinerea* *Colletotrichum lindemuthianum*	855.1 nM 6.9 μM 87.6 μM	Shentu et al. (2014)

(continued)

Table 11.2 (continued)

Fungal endophyte taxa	Medicinal Plant/ Tissue	Compounds isolated	Biological activity	EC_{50}/ IC_{50}/MIC	Reference
Xylaria sp.	*Toona sinensis*/ leaves	**55.** Cytochalasin Z_{27} ($C_{28}H_{33}NO_6$) **56.** Cytochalasin Z_{28} ($C_{28}H_{33}NO_6$) **57.** *seco*-Cytochalasin E ($C_{29}H_{37}NO_8$) **58.** Cytochalasin Z_{18} ($C_{31}H_{43}NO_9$) **59.** Cytochalasin E ($C_{28}H_{33}NO_7$)	*Gibberella saubinetti*	**55.** 50 μM **56.** 12.5 μM **57.** 100 μM **58.** >100 μM **59.** 100 μM	Zhang et al. (2014b)
			Alternaria solani	**55.** 50 μM **56.** 50 μM **57.** 100 μM **58.** 50 μM **59.** 50 μM	
			Botrytis cinerea	**55.** 100 μM **56.** 100 μM **57.** 100 μM **58.** >100 μM **59.** 100 μM	
			Fusarium solani	**55.** 100 μM **56.** 50 μM **57.** >100 μM **58.** 100 μM **59.** >100 μM	
Botryosphaeria dothidea	*Melia azedarach*/ bark	**60.** Pycnophorin ($C_{27}H_{40}O_4$) **61.** Stemphyperylenol ($C_{20}H_{16}O_6$) **62.** Chaetoglobosin C ($C_{32}H_{36}N_2O_5$) **63.** Djalonensone ($C_{15}H_{12}O_5$) **64.** Alternariol ($C_{14}H_{10}O_5$) **65.** 5′-methoxy-6-methylbiphenyl-3,4,3′-triol ($C_{14}H_{14}O_4$)	*Botrytis cinerea*	**60.** 100 μM **61.** 100 μM **62.** 200 μM **63.** 25 μM **64.** NA **65.** NA **66.** 100 μM **67.** 100 μM **68.** 50 μM	Xiao et al. (2014a)

		66. *β*-sitosterol glucoside **67.** 5-(hydroxymethyl)-1*H*-pyrrole-2-carbaldehyde ($C_6H_7NO_2$) **68.** 5-hydroxymethylfurfural ($C_6H_6O_3$)	*Alternaria solani*	**60.** 6.3 μM **61.** 1.6 μM **62.** 12.5 μM **63.** 25 μM **64.** 12.5 μM **65.** 50 μM **66.** 6.3 μM **67.** 50 μM **68.** 6.3 μM	
Pezicula sp.	*Forsythia viridissima*/twigs	**69.** Mellein ($C_{10}H_{10}O_3$)	*Botrytis cinerea*, *Colletotrichum orbiculare*, *Verticillium dahliae*, *Fusarium oxysporum* f. sp. *cucumerinum*, *Pyricularia oryzae*, *Pestalotia diospyri*, *Pythium ultimum*, *Sclerotinia sclerotiorum*, *Fulvia fulva*	272.9 μM - 846.9 μM - 916.9 μM - 892.9 μM - 666.9 μM - 903.8 μM - 703.6 μM - 1.2 mM - 258.1 μM	Wang et al. (2014)

(continued)

Table 11.2 (continued)

Fungal endophyte taxa	Medicinal Plant/ Tissue	Compounds isolated	Biological activity	EC_{50}/ IC_{50}/MIC	Reference
Xylaria sp.	*Azadirachta indica*/stem	**70.** (1*S*,4*S*,5*R*,7*R*,10*R*,11*R*)-Guaiane-5,10,11,12-tetraol ($C_{15}H_{28}O_4$) **71.** (1*S*,4*S*,5*S*,7*R*,10*R*,11*S*)-Guaiane-1,10,11,12-tetraol ($C_{15}H_{28}O_4$) **72.** (1*S*,4*S*,5*R*,7*R*,10*R*,11*S*)-Guaiane-5,10,11,12-tetraol ($C_{15}H_{28}O_4$) **73.** (1*S*,4*S*,5*S*,7*R*,10*R*,11*R*)-Guaiane-1,10,11,12-tetraol ($C_{15}H_{28}O_4$) **74.** (1*R*,3*S*,4*R*,5*S*,7*R*,10*R*,11*S*)-Guaiane-3,10,11,12-tetraol ($C_{15}H_{28}O_4$) **75.** (1*R*,3*R*,4*R*,5*S*,7*R*,10*R*,11*R*)-Guaiane-3,10,11,12-tetraol ($C_{15}H_{28}O_4$) **76.** (1*R*,4*S*,5*S*,7*S*,9*R*,10*S*,11*R*)-Guaiane-9,10,11,12-tetraol ($C_{15}H_{28}O_4$) **77.** (1*R*,4*S*,5*S*,7*R*,10*R*,11*S*)-Guaiane-10,11,12-triol ($C_{15}H_{28}O_3$) **78.** (1*R*,4*S*,5*S*,7*R*,10*R*,11*R*)-Guaiane-10,11,12-triol ($C_{15}H_{28}O_3$) **79.** 14α,16-Epoxy-18-norisopimar-7-en-4α-ol ($C_{19}H_{30}O_2$) **80.** 16-O-Sulfo-18-norisopimar-7-en-4α,16-diol ($C_{19}H_{32}O_5S$) **81.** 9-deoxy-hymatoxin A ($C_{20}H_{30}O_6S$)	*Candida albicans*	**70.** 939.9 μM **71.** 117.5 μM **72.** 469.9 μM **73.** 234.9 μM **74.** 234.9 μM **75.** 469.9 μM **76.** 117.5 μM **77.** 499.3 μM **78.** 499.3 μM **79.** 220.4 μM **80.** 171.8 μM **81.** 40.2 μM	Wu et al. (2014)

			Aspergillus niger	**70.** 469.9 µM **71.** 234.9 µM **72.** 939.9 µM **73.** 234.9 µM **74.** 1.9 mM **75.** 1.9 mM **76.** 469.9 µM **77.** 998.5 µM **78.** >2 mM **79.** 220.4 µM **80.** 343.6 µM **81.** 80.3 µM	
			Pyricularia oryzae	**70.** 939.9 µM **71.** 939.9 µM **72.** 469.9 µM **73.** 939.9 µM **74.** 939.9 µM **75.** 469.9 µM **76.** 1.87 mM **77.** 1.99 mM **78.** 998.5 µM **79.** 881.4 µM **80.** 85.9 µM **81.** 40.1 µM	

(continued)

Table 11.2 (continued)

Fungal endophyte taxa	Medicinal Plant/ Tissue	Compounds isolated	Biological activity	EC_{50}/ IC_{50}/MIC	Reference
			Fusarium avenaceum	**70.** 1.9 mM **71.** >1.9 mM **72.** 1.9 mM **73.** >1.9 mM **74.** >1.9 mM **75.** 1.9 mM **76.** >1.9 mM **77.** >2 mM **78.** 2 mM **79.** 220.4 µM **80.** 343.6 µM **81.** 160.6 µM	
			Hormodendrum compactum	**70.** 469.9 µM **71.** 235 µM **72.** 939.9 µM **73.** 939.9 µM **74.** 469.9 µM **75.** 469.9 µM **76.** 939.9 µM **77.** 499.3 µM **78.** 998.5 µM **79.** 440.7 µM **80.** 171.8 µM **81.** 160.6 µM	
Bipolaris sp.	*Gynura hispida*/ leaf	**82.** Bipolamides A ($C_{18}H_{29}NO_4$) **83.** Bipolamides B ($C_{12}H_{19}NO$)	*Candida albicans* OUT 6266	**82.** >395.8 µM **83.** 662.3 µM	Siriwach et al. (2014)
			Aspergillus niger ATCC 6275	**82.** >395.8 µM **83.** 331.1 µM	

			Rhizopus oryzae ATCC 10404	**82.** >395.8 μM **83.** 331.1 μM	
			Geotrichum candidum IFO 4598	**82.** >395.8 μM **83.** >662.3 μM	
			Cladosporium cladosporioides FERMS-9	**82.** >395.8 μM **83.** 82.8 μM	
			Alternaria mali NBRC 8984	**82.** >395.8 μM **83.** >662.3 μM	
			Cladosporium cucumerinum NBRC 6370	**82.** >395.8 μM **83.** 165.6 μM	
			Fusarium oxysporum NBRC 31224	**82.** >395.8 μM **83.** 662.3 μM	
Berkleasmium sp.	*Dioscorea zingiberensis*/ rhizomes	**84.** Diepoxin ζ ($C_{20}H_{14}O_7$) **85**. Palmarumycin C11 ($C_{20}H_{14}O_5$) **86**. Palmarumycin C12 **87**. Cladospirone B ($C_{20}H_{14}O_6$) **88**. Palmarumycin C6 **89**. 1,4,7β-trihydroxy8(spirodioxy-1′,8′-naphthyl)-7,8-dihydronaphthalene **90**. Palmarumycin C8 ($C_{20}H_{13}ClO_6$)	*Magnaporthe oryzae*	**84**. 286.6 μM **85**. 96.6 μM **86**. 76.7 μg mL^{-1} **87**. 185 μM **88**. 124.5 μg mL^{-1} **89**. 35.9 μg mL^{-1} **90**. 23.7 μM	Shan et al. (2014)
Exserohilum sp.	*Acer truncatum*/ leaf	**91.** Exserolide C ($C_{16}H_{20}O_6$) **92.** (12*R*)-12-hydroxymonocerin	*Fusarium oxysporum*	**91.** 64.9 μM **92.** 20 μg mL^{-1}	Li et al. (2014)

(continued)

Table 11.2 (continued)

Fungal endophyte taxa	Medicinal Plant/ Tissue	Compounds isolated	Biological activity	EC_{50}/ IC_{50}/MIC	Reference
Aspergillus sp.	*Melia azedarach*/ stem bark	**93.** Dianhydro-aurasperone C ($C_{31}H_{24}O_{10}$) **94.** Isoaurasperone A ($C_{32}H_{26}O_{10}$) **95.** Fonsecinone A ($C_{32}H_{26}O_{10}$) **96.** Asperpyrone A ($C_{31}H_{24}O_{10}$) **97.** Asperazine ($C_{40}H_{36}N_6O_4$) **98.** Rubrofusarin B ($C_{15}H_{12}O_5$) **99.** (*R*)-3-hydroxybutanonitrile	*Gibberella saubinetti*	**93.** NA **94.** 25 μM **95.** 50 μM **96.** 25 μM **97.** 25 μM **98.** NA **99.** 12.5 μM	Xiao et al. (2014b)
			Magnaporthe grisea	**93–94.** NA **95.** 12.5 μM **96–98.** NA **99.** 25 μM	
			Botrytis cinerea	**93–94.** NA **95.** 25 μM **96.** NA **97.** 50 μM **98–99.** NA	
			Colletotrichum gloeosporioides	**93.** NA **94.** 25 μM **95–98.** NA **99.** 50 μM	
			Alternaria solani	**93.** NA **94.** 12.5 μM **95–97.** 6.3 μM **98.** 12.5 μM **99.** 25 μM	

Trichothecium sp.	*Phyllanthus amarus*/leaf	**100.** Trichothecinol-A ($C_{19}H_{24}O_6$)	*Saccharomyces cerevisiae* *Cryptococcus albidus* var. *diffluens* NCIM 3371 *Cryptococcus albidus* var. *diffluens* NCIM 3372 *Fusarium oxysporum* *Aspergillus flavus* *Trichoderma reesei* *Penicillium expansum* *Trichoderma viride* *Paecilomyces variotii* *Aspergillus niger*	49.3 μM 11.7 μM 7.17 μM 20.6 μM 69.7 μM 43.1 μM 19 μM 105.1 μM 24.9 μM 26.2 μM	Taware et al. (2014)
Pestalotiopsis mangiferae	*Mangifera indica*/leaves	**101.** 4-(2,4,7-trioxa-bicyclo[4.1.0] heptan-3-yl) phenol ($C_{10}H_{10}O_4$)	*Candida albicans*	200.8 nM	Subban et al. (2013)
Chaetomium globosum	*Ginkgo biloba*/leaf	**102.** 1,2-benzenedicarboxaldehyde-3,4,5-trihydroxy-6-methyl (flavipin) ($C_9H_8O_5$)	*Fusarium graminearum* *Sclerotinia sclerotiorum* *Rhizoctonia solani* *Alternaria solani* *Phytophthora capsici*	3.7 μM 18.8 μM 13.4 μM 63 μM 14.1 μM	Xiao et al. (2013)
Chaetomium cupreum	*Macleaya cordata*	**103.** Ergosta-5,7,22-trien-3beta-ol ($C_{28}H_{44}O$)	*Sclerotinia sclerotiorum* *Botrytis cinerea*	315.2 μM 479 μM	Wang et al. (2013)
Pestalotiopsis fici	*Camellia sinensis*/branches	**104..** Ficipyrone A ($C_{14}H_{22}O_5$)	*Gibberella zeae*	15.9 μM	Liu et al. (2013)
Xylaria feejeensis	*Croton lechleri*/ stem	**105.** (4*S*,7*S*,8*S*,9*R*)-4-*O*-succinyl-7,8-dihydroxy-9-heptyl-nonen-9-olide (xyolide) ($C_{20}H_{32}O_8$)	*Pythium ultimum*	425 μM	Baraban et al. (2013)

(continued)

Table 11.2 (continued)

Fungal endophyte taxa	Medicinal Plant/ Tissue	Compounds isolated	Biological activity	EC_{50}/ IC_{50}/MIC	Reference
Phomopsis sp.	*Aconitum carmichaeli*/ stems	**106.** (14*β*,22*E*)-9,14-dihydroxyergosta-4,7,22-triene-3,6-dione ($C_{28}H_{40}O_4$) **107.** (5*α*,6*β*,15*β*,22*E*)-6-ethoxy-5,15-dihydroxyergosta-7,22-dien-3-one ($C_{30}H_{48}O_4$) **108.** Calvasterol A ($C_{28}H_{38}O_3$) **109.** Calvasterol B ($C_{28}H_{40}O_4$) **110.** Ganodermaside D ($C_{28}H_{40}O_2$)	*Candida albicans*	**106.** 145.3 μM **107.** 270.8 μM **108.** >1.2 mM **109.** 290.5 μM **110.** 1.3 mM	Wu et al. (2013a)
			Aspergillus niger	**106.** 290.5 μM **107.** >1.1 mM **108.** 605.8 μM **109.** 581 μM **110.** 626.5 μM	
			Pyricularia oryzae	**106.** >1.2 mM **107.** >1.1 mM **108.** 302.9 μM **109.** >1.2 mM **110.** >1.3 mM	
			Fusarium avenaceum	**106.** 1.2 mM **107.** 270.8 μM **108.** 151.4 μM **109.** 1.2 mM **110.** 156.6 μM	
			Hormodendrum compactum	**106.** 145.3 μM **107.** 541.6 μM **108.** >1.2 mM **109.** 581 μM **110.** 313.3 μM	

			Trichophyton gypseum	**106.** >1.2 mM **107.** 1.08 mM **108.** 605.8 μM **109.** >1.7 μM **110.** 1.3 mM	
Aspergillus sp.	*Gloriosa superba*/seeds	**111.** 6-Methyl-1,2,3-trihydroxy-7,8-cyclohepta-9,12-diene11-one-5,6,7,8-tetralene-7-acetamide ($C_{18}H_{19}NO_5$)	*Saccharomyces cerevisiae* *Candida albicans* *Cryptococcus gastricus*	75.9 μM 38 μM 151.8 μM	Budhiraja et al. (2013)
Hyalodendriella sp.	Hybrid 'Neva' (*Populus deltoides* Marsh × *P. nigra* L)/ stems	**112.** 2-chloro-3,7-dihydroxy-9-methoxy-1-methyl-6*H*-dibenzo[*b*,*d*]pyran-6-one (palmariol B) ($C_{15}H_{11}ClO_5$) **113.** 4,8-dihydroxy-3-methylbenzopyran-1-one (4-hydroxymellein) ($C_{10}H_{10}O_4$) **114.** 3,7-dihydroxy-9-methoxy-1-methyl-6*H*-dibenzo [*b*,*d*]pyran-6-one (alternariol 9-methyl ether) ($C_{15}H_{12}O_5$) **115.** 1,7-dihydroxy-3,9-dimethoxy-4a-methyl-6*H*-dibenzo[*b*,*d*]pyran-2,6 (4a*H*)- dione (botrallin) ($C_{16}H_{14}O_7$)	*Magnaporthe oryzae*	**112.** 387.1 μM **113.** 584 μM **114.** 452.5 μM **115.** 336.8 μM	Meng et al. (2012)
Alternaria sp.	*Trixis vauthieri*/ leaf	**116.** Altenusin ($C_{15}H_{14}O_6$)	Eleven clinical *Paracoccidioides brasiliensis* strains	6.5–107.5 μM	Johann et al. (2012)

NA: inactive. EC_{50} - Half maximal effective concentration. IC_{50} - Half maximal inhibitory concentration. MIC - Minimum inhibitory concentration.

bioactive culture which, after threefold dilution, completely inhibited in vitro the mycelial growth and conidia germination of *F. graminearum.* The in vivo protective efficacy of the diluted broth was 54.9% and its curative efficacy 48.8%. Bioassay-guided fractionation resulted in the isolation of 1,2-benzenedicarboxaldehyde-3,4,5-trihydroxy-6-methyl (flavipin) (**102**) that inhibited the growth of the plant-pathogenic fungi *F. graminearum* (EC_{50} value of 3.7 μM), *S. sclerotiorum* (EC_{50} value of 18.8 μM), *Rhizoctonia solani* (EC_{50} value of 13.4 μM), *P. capsici* (EC_{50} value of 14.1 μM), and *Alternaria solani* (EC_{50} value of 63 μM) (Xiao et al. 2013). In a more recent work, Zhao et al. (2017) reinvestigated *Chaetomium globosum* CDW7 and reported the isolation of six known compounds, namely, chaetoglobosins **A**–**E** and **Vb**. Chaetoglobosins **A** (**7**) and **D** (**8**) exhibited inhibitory activity against *S. sclerotiorum* with IC_{50} values of 0.6 and 1.2 μM, respectively (Zhao et al. 2017).

Zhang et al. (2013) studied the endophytic fungi *Chaetomium globosum*, associated with *G. biloba* growing in China, and isolated the alkaloids chaetoglobosins A, C, D, E, G, and R (**120**–**125**) along with ergosterol (**126**), allantoin (**127**), and uracil (**128**). Chaetoglobosins A, C, D, E, G, and R (**120**–**125**) showed significant growth inhibitory activity against the phytopathogenic fungi *Rhizopus stolonifer* and *Coniothyrium diplodiella* at a concentration of 20 μg/disc. Cao et al. (2016) reported that *Nodulisporium* sp. A2, associated with leaves of *G. biloba*, as producer of the sporothriolide (**129**), a metabolite produced by the fungus, showed potent antifungal activity against *Rhizoctonia solani* and *Sclerotinia sclerotiorum* and inhibits conidium germination of *Magnaporthe oryzae* in vitro and in vivo.

Xu et al. (2016) described a new monoterpene lactone (3*R*,4*R*,6*R*,7*S*)-7-hydroxyl-3,7-dimethyl-oxabicyclo[3.3.1]nonan-2-one (**25**) and the known compound (3*R*, 4*R*)-3-(7-methylcyclohexenyl)-propanoic acid (**26**) from *Pestalotiopsis foedan*, an endophyte fungus obtained from the branch of *Bruguiera sexangula* occurring in China. Both compounds exhibited strong antifungal activities against *B. cinerea* and *Phytophthora nicotianae* with MIC values of 3.1 and 6.3 μg mL^{-1}, respectively, values close to the MIC of the antifungal drug control ketoconazole (3.1 μg mL^{-1}). Compound **26** also displayed modest antifungal activity against *C. albicans,* with a MIC value of 50 μg mL^{-1} (Xu et al. 2016).

Bioassay-guided fractionation of the endophytic fungus *Phoma* sp., isolated from roots of *Eleusine coracana*, resulted in the identification of four antifungal compounds, 3-hydroxy-4-(3-hydroxyphenyl)-2-quinolonemonohydrate (viridicatol alkaloid) (**130**), 3-acetyl-5-sec-butyltetramicacid (tenuazonic acid) (**131**), alternariol **(132)**, and alternariol-5-O-methyl ether or djalonensone(3,7-dihydroxy-9-methoxy-1-methyl-6H-dibenzo[b,d]pyran-6-one) (**133**). The antifungal activity of the compounds **130**–**133** was evaluated using the agar disc diffusion method (20 μl of 5 mg mL^{-1}) and produced growth inhibition zones of 1.8, 2, 1.5, and 1.5 mm, respectively (Mousa et al. 2015). The extract of the endophytic *Seimatosporium* sp., isolated from *Salsola oppositifolia* (Spain), was further purified to give pure new compound, 5,6,7,8-tetrahydro-1,5-dihydroxy-3-methoxy-8-oxonaphthalene-2-carbaldehyde (seimatorone) (**134**), and the known compounds, 1-(2,6-dihydroxyphenyl)-3-hydroxybutan-1-one (**135**), 1-(2,6-dihydroxyphenyl)butan-1-one (**136**), 1-(2-hydroxy-6-methoxyphenyl) butan-1-one (**137**), 5-hydroxy-2-methyl-4*H*-chromen-4-one (**138**), 2,3-dihydro-5-hy-

droxy-2-methyl-4*H*-chromen-4-one (**139**), 8-methoxynaphthalen-1-ol (**140**), nodulisporin A (**141**), nodulisporin B (**142**), and daldinol (**143**). Seimatorone demonstrated antifungal activity against *Microbotryum violaceum* in the agar diffusion assay with partial inhibition, once there was some growth within the zone of inhibition (Hussain et al. 2015).

Chapla et al. (2014a) characterized the new compound, 2-phenylethyl 1*H*-indol-3-yl-acetate (**144**), and seven known compounds, uracil (**145**), cyclo-(*S**-Pro-*S**-Tyr) (**146**), cyclo-(*S**-Pro-*S**-Val) (**147**), 2(2-aminophenyl)-acetic acid (**148**), 2(4-hydroxyphenyl)acetic acid (**149**), 4-hydroxybenzamide (**150**), and 2-(2-hydroxyphenyl)-acetic acid (**151**), from the endophytic fungus *Colletotrichum gloeosporioides* associated with leaves of *Michelia champaca* (*Magnoliaceae*) growing in São Paulo, Brazil. All compounds were evaluated for their antifungal activities against two phytopathogenic fungi, *C. cladosporioides* and *C. sphaerospermum*, using the Thin-layer chromatography (TLC) diffusion method at 100 μg/spot and nystatin at 1 μg/spot as positive control. Compounds **144**, **150,** and **151** exhibited activity against both fungal species, while compound **149** was highly active against *C. cladosporioides* but showed only moderate activity on *C. sphaerospermum*. When compounds **144**, **149**, **150,** and **151** were evaluated at doses ranging from 1 to 100 μg/spot, **144** exhibited potent antifungal activity at 5 μg, which was similar to that observed for the positive control (nystatin), demonstrating the potential of **144** as an antifungal agent. Compounds **149**, **150,** and **151** exhibited moderate antifungal activity at 25 μg (Chapla et al. 2014a).

The ethyl acetate extract of endophytic fungus *Coniothyrium* sp., isolated from *Salsola oppositifolia* (Canary Islands), afforded the known hydroxyanthraquinones, pachybasin (**152**), 1,7-dihydroxy-3-methyl-9,10-anthraquinone (**153**), phomarin (**154**), and 1-hydroxy-3-hydroxymethyl-9,10-anthraquinone (**155**), together with four new derivatives having a tetralone moiety, namely, coniothyrinones A–D (**156**–**159**). When tested in the agar diffusion assay (0.05 mg) on *Microbotryum violaceum*, *B. cinerea*, and *Septoria tritici*, compounds **154**, **155**, and **156** showed strong antifungal activity against *M. violaceum* (10, 8, and 7.5 mm of the zone of inhibition, respectively) and *B. cinerea* (9, 9, and 12.5 mm of the zone of inhibition, respectively) (Sun et al. 2013).

Huang et al. (2015) obtained five new guaiane sesquiterpenes **49–53** from the culture broth of the endophytic fungus *Xylaria* sp. YM 311647, which were isolated from *Azadirachta indica*. The compounds were evaluated against the pathogenic fungi *C. albicans*, *A. niger*, *P. oryzae*, *F. avenaceum*, and *Hormodendrum compactum* by means of the broth microdilution method. All compounds exhibited moderate or weak antifungal activities against *P. oryzae* and *H. compactum* with MIC values varying from 111 to 939.9 μM, with compound **52** being the most active against *P. oryzae*. Compounds **51** and **52** exhibited moderate antifungal activities against *H. compactum* with MIC value 221.9 μM. In addition, compounds **52** and **53** showed the most potent antifungal activities against *C. albicans* with MIC values of 110.96 and 111.7 μM, respectively. Compound **51** showed moderate inhibitory activities against *C. albicans*, *A. niger*, and *H. compactum* with MIC value of

221.9 μM. None of the compounds showed activity against *F. avenaceum* (Huang et al. 2015).

Two new tetranorlabdane diterpenoids, named botryosphaerin G (**160**) and H (**161**), along with seven known tetranorlabdane diterpenes, 13,14,15,16-tetranorlabd-7-en-19,6β:12,17-diolide (**162**), botryosphaerin A (**163**), 3a,10b-dimethyl-1,2,3,3a,5a,7,10b,10c-octahydro-5,8-dioxa-acephenanthrylene-4,9-dione (**164**), acrostalidic acid (**165**), botryosphaerin B (**166**), LL-Z1271β (**167**), and acrostalic acid (**168**), were isolated from the endophytic fungus *Botryosphaeria* sp. P483 associated with the Chinese medicinal plant *Huperzia serrata*. Compounds **161** and **162** showed antifungal activity against phytopathogenic fungi *Gaeumannomyces graminis*, *Fusarium moniliforme*, *F. solani*, *F. oxysporum*, and *Pyricularia oryzae* using the disk diffusion method at 100 μg/disk (Chen et al. 2015).

Pereira et al. (2015) demonstrated that the crude extract of the endophytic fungus *Mycosphaerella* sp. UFMGCB 2032, recovered from *Eugenia bimarginata* (Brazil), exhibited outstanding antifungal activity against *Cryptococcus neoformans* and *C. gattii*, with MIC values of 31.2 μg mL^{-1} and 7.8 μg mL^{-1}, respectively. The fractionation of this extract afforded two eicosanoic acids, (2*S*,3*R*,4*R*)-(*E*)-2-amino-3,4-dihydroxy-2-(hydroxymethyl)-14-oxoeicos-6,12-dienoic acid (**47**) with MIC values of 3.3 and 6.3 μM against *C. neoformans* and *C. gattii*, respectively, and myriocin (**48**), with MIC values of 1.24 μM to both targets. Nalli et al. (2015) reported the identification of four new bioactive metabolites, phialomustin A–D (**169–172**), isolated from the endophytic fungus *Phialophora mustea* associated with the corms of *Crocus sativus.* Compounds **171** and **172** showed antifungal activities against *C. albicans* with IC_{50} values of 14.3 and 73.6 μM, respectively. Compound **171** was active against *A. fumigatus*, *A. parasiticus*, and *A. flavus* with IC_{50} values of 60.6, 35.2, and 84.4 μM, respectively (Nalli et al. 2015).

The chemical evaluation of the crude extract of the endophytic *Guignardia* sp., from *Euphorbia sieboldiana* leaves, led to the isolation of nine new meroterpenes, guignardones J-L (**173–175**), 13-hydroxylated guignardone A (**176**), 12-hydroxylated guignardone A (**177**), 17-hydroxylated guignardone A (**178**), guignardones M-O (**179–181**), and a new dioxolanone derivative, 10-hydroxylated guignardianone C (**182**), together with seven known compounds, guignardones A-C (**183–185**), guignardones G and H (**186–187**), guignardic acid (**188**), and palmarumycin C11 (**189**). The compounds were evaluated for their inhibitory effects alone and with fluconazole on the growth and biofilms of *C. albicans*. At 6.3 μg mL^{-1} concentration, combined with 0.031 μg mL^{-1} of fluconazole, compounds **180** and **188** showed inhibition on the growth of *C. albicans* with fractional inhibitory concentration index values of 0.2 and 0.2, respectively (Li et al. 2015b).

Altenusin (**190**), isoochracinic acid (**191**), altenuic acid (**192**), and 2,5-dimethyl-7-hydroxychromone (**193**) were isolated from *Alternaria alternata* associated with *Terminalia chebula* (Thailand). All compounds were investigated for their activity on *Candida albicans* using disc diffusion assay. Altenusin (**190**) exhibited weak activity against *C. albicans* with an unclear inhibition zone diameter of 8.3 mm (at the concentration of 256 μg/disc). In the presence of a subinhibitory concentration

of ketoconazole at 0.1 μg mL^{-1}, altenusin produced a clear inhibition zone diameter of 19.2 mm (Phaopongthai et al. 2013).

Li et al. (2014) obtained six new isocoumarin derivatives, exserolides A–F (**194**–**199**), together with four known metabolites, monocerin (**200**), 11-hydroxymonocerin (**201**), (12R)–(**202**), and (12S)-12-hydroxymonocerin (**203**). They were isolated from the ethyl acetate (EtOAc) extract of endophytic fungus *Exserohilum* sp., recovered from the leaves of *Acer truncatum* (China). All the compounds were tested for their antifungal activity against the plant pathogenic fungus *F. oxysporum*. Compounds **196** and **202** displayed MIC value of 20 μg mL^{-1}, while the positive control amphotericin B showed a MIC value of 0.6 μg mL^{-1} (Li et al. 2014). Two compounds named *cis*-4-acetoxyoxymellein (**204**) and 8-deoxy-6-hydroxy-*cis*-4-acetoxyoxymellein (**205**) were identified by Hussain et al. (2014) from an unidentified endophytic fungus isolated from *Meliotus dentatus*. Both compounds showed significant antifungal effect toward *M. violaceum*, *B. cinerea*, and *Septoria tritici* when tested in the agar diffusion assay.

Carvalho et al. (2016) reported the identification of the compounds (−)-5-methylmellein (**206**) and (−)-(3*R*)-8-hydroxy-6-methoxy-3,5-dimethyl-3,4-dihydroisocoumarin (**207**) from the endophytic *Biscogniauxia mediterranea* EPU38CA associated with *Echinacea purpurea* (USA). The compounds were evaluated against plant pathogenic fungi at a dose of 300 μM, with the compound **206** showing weak activity against *P. obscurans*, *P. viticola*, and *F. oxysporum* with 43.5, 36, and 5% of inhibition, respectively. Using the same methodology, compound **207** showed antifungal activity against *B. cinerea* (58%), *P. viticola* (50%), and *P. obscurans* (70%). *B. mediterranea* was also isolated from the plant *Opuntia humifusa* (USA) by Silva-Hughes et al. (2015) and yielded (−)-5-methylmellein (**208**), a compound that displayed moderate antifungal activity against the phytopathogenic fungi *P. obscurans* (63.5% growth inhibition) and *F. oxysporum* (20.1%).

Kajula et al. (2016) identified three new epithiodiketopiperazine natural products, outovirin A–C (**209**–**211**), produced by the endophytic fungus *Penicillium raciborskii* isolated from *Rhododendron tomentosum*. The authors evaluated the antifungal activity of the compounds against *F. oxysporum*, *B. cinerea*, and *Verticillium dahliae* by microspectrophotometry using a dose-response growth inhibition assay. Outovirin C inhibited growth of all fungal isolates at a low concentration of 0.4 mM, but a more significant growth inhibition was observed at the higher concentration of 0.8 mM. This compound was most active against *B. cinerea* (57% inhibition) and slightly less effective against *V. dahliae* (45% inhibition) (Kajula et al. 2016).

Four new compounds, murranofuran A (**212**), murranolide A (**213**), murranopyrone (**214**), and murranoic acid A (**215**), along with six known metabolites, *N*-(2-hydroxy-6-methoxyphenyl)acetamide (**216**), curvularin (**217**), (*S*)-dehydrocurvularin (**218**), pyrenolide A (**219**), modiolide A (**220**), and 8-hydroxy-6-methoxy-3-methylisocoumarin (**221**), were identified from the *Curvularia* sp., an endophytic fungus isolated from *Murraya koenigii* (Bangladesh). The compounds were subjected to motility, inhibitory, and zoosporicidal activity tests

against *Phytophthora capsici* at different concentration and time-course activities. The most noticeable zoospore motility-inhibitory activity was exhibited by pyrenolide A (**219**), where the highest activity (100%) was achieved at a very low concentration (0.5 μg mL^{-1}) within a short time (30 min). Compounds **213**, **214**, **217**, **218**, **220**, and **221** exhibited zoospore motility impairment activity, but with IC_{50} values in the range 50–100 μg mL^{-1} (Mondol et al. 2017).

Silva et al. (2017a) described the isolation, structure, and antifungal activity of three new isoaigialones, A–C (**222**–**224**), along with aigialone (**225**) from the endophytic fungus *Phaeoacremonium* sp. isolated from leaves of *Senna spectabilis* (Brazil). Using direct bioautography all the compounds were evaluated against *C. cladosporioides* and *C. sphaerospermum*. The compounds **223** and **225** exhibited antifungal activity, with a detection limit of 5 μg/spot, for both species of *Cladosporium*, while compound **224** displayed weak activity (detection limit >5 μg/spot), with a detection limit of 25 μg/spot.

The compounds epicolactone (**226**) and epicoccolides A and B (**227**–**228**), together with seven known metabolites, were obtained from the endophytic fungus *Epicoccum* sp. CAFTBO isolated from *Theobroma cacao*. The compounds **226**–**228** exhibited antifungal activity in the agar diffusion test against *Pythium ultimum* and *Rhizoctonia solani* with MIC values of 20–80 μg/disk (Talontsi et al. 2013).

11.4 Antiviral Compounds

Viral diseases are among the greatest concerns among the infectious diseases. WHO has released a list of priority diseases and pathogens for the year 2018 and among these diseases are Crimean-Congo hemorrhagic fever, Ebola, Zika, and Chikungunya virus (OPAS - OMS 2018). Thus, recent research attempts to identify antiviral compounds to produce vaccines, aiming at an immunization of the population.

As already mentioned, endophytic fungi are a promising source of biologically active secondary metabolites with numerous applications, including the production of antiviral compounds (Pamphile et al. 2017). However, there had been few reports on antiviral metabolites from endophytic fungi, even though those found show promising results (Kaul et al. 2012). Zhang et al. (2011a, b) isolated from the inner shell of *Aegiceras corniculatum* the endophytic fungus *Emericella* sp. that can produce two isoindolone derivatives. These two substances showed moderate antiviral activity with IC_{50} of 42.1 and 62.1 μg mL^{-1}, against influenza A (H_1N_1). *Aegiceras corniculatum* is a plant that grows in mangroves of tropical and subtropical regions. Species of *Aegiceras* are known to be used in the treatment of ulcers, liver damage, asthma, diabetes, and rheumatism and as an anti-inflammatory agent (Roome et al. 2008).

Guo et al. (2000) isolated the endophytic *Cytonaema* sp. from tissues of *Quercus* sp., which was able to produce the cytonic acids A and B and described as having antiviral activity since they are inhibitors of human cytomegalovirus protease, with IC_{50} of 43 μM and 11 μM, respectively. Plants of this genus are used by indigenous

peoples in Canada for the treatment of diabetes and its complications (McCune and Johns 2002).

Hinnuloquinone is another antiviral compound that inhibits human immunodeficiency virus type 1 protease (HIV-1) (Singh et al. 2004; Kumar et al. 2014). This compound had already been isolated from an endophytic fungus associated with the leaves of *Quercus coccifera* (Baker and Satish 2015). *Quercus coccifera* is used for wound healing in the villages of Yunt Mountain in Turkey (Ugurlu and Secmen 2008).

Pullarin A is a compound produced by the endophytic *Pullaria sp.*, which was reported to be associated with the leaves of *Caulophyllum* sp. grown in Thailand. This compound showed antiviral activity with IC_{50} of 3.3 μg mL^{-1} against herpes virus type 1 - HSV-1 (Isaka et al. 2007; Borges et al. 2009).

11.5 Antitumor Compounds

According to the WHO, the number of deaths caused by the diverse types of cancer in the world can reach 8.8 million people annually. Estimates indicate that 14 million people develop cancer every year and by 2030 that number should reach 21 million people (OPAS/OMS 2017). As a result, the search for new treatments has grown significantly throughout the world. The search of anticancer secondary metabolites produced by endophytic fungi associated with medicinal plants has been studied since the discovery of taxol, first isolated from the bark of *Taxus brevifolia* in 1971. Taxol has proven efficacy against prostate, ovarian, breast, and lung cancers (Zhao et al. 2010b; Manju et al. 2012). Interestingly, taxol was also found in *Taxomyces andreanae,* an endophytic fungus isolated from the bark of *T. brevifolia*. Other studies demonstrated that taxol can be produced by endophytic fungi isolated from other plants (Pandi et al. 2011). Qiao et al. (2017), for example, isolated the taxol from the endophytic fungus *Aspergillus aculeatinus*, isolated from the inner and outer bark of the plant *Taxus chinensis* var. *mairei*. The endophytic fungus *Cladosporium* sp., isolated from the leaves and stem of the *Taxus baccata* plant in the northern forest of Iran, was also able to produce taxol (Kasaei et al. 2017). Taxol prevents tubulin molecules from depolymerizing during cell division processes. This happens because this compound inhibits cell replication and migration, stopping the cycle of division of mitosis in late phase G2 (Strobel and Daisy 2003; Yang and Horwitz 2017).

Camptotheca acuminata is a plant native to central China and widely used in the popular medicine. This species is rich in camptothecin (Lin et al. 2007), an anticancer compound that acts on the enzyme topoisomerase I which is responsible for the relaxation or not of the DNA molecule during the processes of replication and transcription (Kusari et al. 2009). It was later found that the endophytic fungus *Fusarium solani*, originating from the inner bark of this *C. acuminata* was also able to produce camptothecin (Kusari et al. 2012). Moreover, there are also reports of its production by other endophytic fungi associated with other plant species, for example, the

endophytic fungus *Entrophospora infrequens* isolated from the inner bark of *Nothapodytes foetida syn. N. nimmoniana* (Gowda et al. 2002; Puri et al. 2005). This plant, growing on the west coast of India, is used as anticancer, antimalarial, bactericidal, antioxidant, anti-inflammatory, and fungicidal, to treat anemia and HIV infections (Khan et al. 2013). Su et al. (2014) isolated camptothecin from the endophytic fungi *Alternaria alternata*, *C. gloeosporioides*, *Fusarium nematophilum*, and *Phomopsis vaccinia*, all isolated from the leaves, twigs, and roots of *C. cuminata*. From this plant yet another endophytic fungus, *Fusarium solani*, also produces camptothecin (Ran et al. 2017).

Podophyllum hexandrum is a plant that lives in high altitudes and is native to alpine and subalpine areas of the Himalayas. It has been used since antiquity in traditional Indian and Chinese medicine to treat metabolic imbalance. More recently, its activity against monocytic leukemia, Hodgkin's and non-Hodgkin's lymphomas, bacterial and viral infections, venereal warts, rheumatoid arthralgia associated with limb numbness, and different types of cancer, such as brain, lung, and bladder, has been described (Chawla et al. 2005). *Podophyllum hexandrum* produces a substance called podophyllotoxin that is a precursor to the synthesis of three anticancer compounds: etoposide, teniposide, and etoposide phosphate (You 2005). These compounds inhibit DNA topoisomerase II and are used to treat cancer of the lung, testicles, and some leukemias, among others (Xu et al. 2009; Chandra 2012). Puri et al. (2006) isolated the endophytic fungus *Trametes hirsuta* from the rhizomes of *P. hexandrum*, which was able to produce podophyllotoxin under laboratory conditions. It has also been isolated from the endophytic *Fusarium oxysporum* associated with the plant *Juniperus recurva* (Kour et al. 2008). *Phialocephala fortini*, an endophytic fungus associated with *Podophyllum peltatum*, also produces this substance. In India, this plant is used in the treatment of snakebite, cancer, vermifuge, and ulcers (Eyberger et al. 2006; Silva et al. 2017b). Podophyllotoxin was also isolated from the endophytic *Fusarium solani* isolated from the root of the plant *P. hexandrum* (Nadeem 2012).

Ergoflavine is an anticancer compound isolated from the Indian medicinal plant *Mimusops elengi* (Kaul et al. 2012). All parts of this plant are known to have medicinal properties. The fruits are used for chronic dysentery and constipation; the flowers relieve headaches and are used against ulcer; and the bark is used to increase fertility in women and also has activity against ulcer (Verekar et al. 2017). Deshmukh et al. (2009) isolated from the leaves of *M. elengi* an endophytic fungus that was shown to produce ergoflavine, significantly active against the proliferation of pancreatic, renal, and lung cancer cells.

Cytochalasins are a large group of secondary metabolites produced by various species of fungi, comprising about 60 different compounds. The first cytochalasins to be studied were A and B. They inhibit actin, sugar uptake, and blocks ion channels (Goietsenoven et al. 2011). Pongcharoen et al. (2006) isolated cytokinins produced by the endophytic fungus *Eutypella scoparia* associated with the plant *Garcinia dulcis*. In Thailand, *G. dulcis* leaves are used for the treatment of inflammation in the lymphatic, mumps, and goiter ducts (Abu et al. 2015). Wagenaar et al. (2000) also report the production of cytochalasins by another endophytic fungi,

Rhinocladiella sp., isolated from *Tripterygium wilfordii*. This plant is endemic in southern China and used to treat immune and inflammatory diseases (OuYang et al. 2007). Caetoglobesins are cytochlasin arrays, and many of them are toxic to human cancer cell lines. More than 40 have been identified and many of them are produced by fungi (Zhang et al. 2010). Caetoglobosin U, a secondary metabolite of the endophytic fungus *Chaetomium globosum*, isolated from the medicinal plant *Imperata cylindrica*, used in the treatment of dysentery and urinary tract infections, was shown to display anticancer activity (Ding et al. 2006; Krishnaiah et al. 2009). Caetoglobesins C, E, and F, among others, were also isolated from this fungal species, but this time isolated from the *G. biloba* plant (Li et al. 2014). The seeds of this plant are used for the treatment of asthma and cough and the leaves are used for heart problems and skin infections (Mahadevan and Park 2008).

Vincristine is another anticancer compound and acts by disrupting mitosis by binding to tubulin dimers, inhibiting the assembly of microtubules (Aly et al. 2010). Kumar et al. (2013) isolated vinscritin from the culture of endophytic fungus *Fusarium oxysporum*, which was associated with the medicinal plant *Catharanthus roseus*. The roots of this plant are used to control blood pressure, and this characteristic is related to the alkaloids present in it. Table 11.3 shows other anticancer compounds isolated from endophytic fungi of medicinal plants in the last 8 years.

11.6 Acetylcholinesterase Inhibitors

Alzheimer's disease (AD) is an age-related neurodegenerative disease with cognitive and neuropsychiatric manifestations that result in progressive disability (Zhao and Tang 2002). According to Alzheimer's Disease International (ADI), 47 million people lived with dementia in the world in 2016 and this number can increase to more than 131 million by 2050 as populations age. That can be related to the AD that lead to a progressive decline in cognitive function that is substantially increased among people aged 65 years or more (Prince et al. 2016).

Cholinesterase inhibitors are important substances recommended for the treatment of cognitive deficits and associated behavioral abnormalities in patients with mild-to-moderate AD (Weinstock 1999; Ballard 2002). The cholinesterase inhibitors can inactivate the enzyme acetylcholinesterase (AChE), preventing the inactivation of acetylcholine (Ach) after its release from the neuron, increasing its ability to stimulate nicotinic and muscarinic receptors (Weinstock 1999; Zhao and Tang 2002). There are no available treatments that stop or reverse the progression of the disease, fact that reinforces the importance of developing medicines that would at least slow the progression of the symptoms (Duthey 2013).

Oliveira et al. (2011) reported the AChE inhibition of (3R,4R)-3,4-dihydro-4,6-dihydroxy-3-methyl-1-oxo-1H-isochromene-5-carboxylic acid produced by the fungus *Xylaria* sp., isolated from the plant *Piper aduncun* with minimum amount required for inhibition of 3 μg compared with the galantamine used as positive control with minimum amount required for inhibition of 1 μg. Singh et al. (2012)

Table 11.3 Antitumor compounds reported from endophytic fungi from medicinal plants

Fungal endophytic taxa	Medicinal plant/tissue	Compound isolated	Biological activity	IC_{50}	Reference
Aspergillus glaucus	*Ipomoea batatas*/leaves	**1.** 2, 14-dihydrox-7-drimen-12, 11-olide	HepG2 MCF-7	229 μM 156.6 μM	Asker et al. (2013)
Penicillium sp.	*Tripterygium wilfordii*/ leaves	**2.** 3-*O*- methylfunicone	KB	90.9 μM	Chen et al. (2014)
Annulohypoxylon squamulosum	*Cinnamomum* sp./stem bark	**3.** (3*S*)-7-hydroxymellein	MCF-7 NCI-H460 SF-268	2.8 μM 3.2 μM 2.9 μM	Cheng et al. (2012)
Aspergillus sp.	*Gloriosa superba*/seeds	**4.** 6- methyl-1,2,3-trihydroxy-7,8-cyclohepta-9,12-diene-11-one-5,6,7,8-tetralene-7-acetamide	MCF-7 THP-1	151.8 μM 91.1 μM	Budhiraja et al. (2013)
Botryotinia fuckeliana	*Ajuga decumbens*/roots	**5.** (12)-cytochalasin	7721 A549 HepG2 MCF-7	0.9 μM 0.7 μM 0.6 μM 0.6 μM	Lin et al. (2015)
Penicillium janthinellum	*Panax ginseng*/roots	**6.** Penicillic acid	MKN45 LOVO A549 MDA-MB-435 HepG2 HL-60	16.7 μM 13 μM 43.8 μM 36.9 μM 21.6 μM 4.7 μM	Zheng et al. (2013)
Alternaria alternata	*Capsicum annuum*/fruits	**7.** Alternariol-10-methyl ether	HL-60 A549 PC-3 HeLa A431 Mia PaCa-2 T47D	85 μM >100 μM > 100 μM > 100 μM 95 μM > 100 μM > 100 μM	Devari et al. (2014)

Chaetomium globosum	*Curcuma wenyujin*/leaves	**8.** Chaetoglobosin X	MFC H22	15.1 μM 7.5 μM	Wang et al. (2012)
Penicillium sp.	*Tripterygium wilfordii*/ leaves	**9.** Deoxyfunicone	KB	22.6 μM	Chen et al. (2014)
Xylaria sp.	*Licuala spinosa*	10. Eremophilanolides 1, 2 e 3	KB MCF-7 NHI-H187	3.8–2 μM	Isaka et al. (2010)
Perenniporia tephropora	*Taxus chinensis var. maire*/ bark	**11.** Ergosterol	HeLa SMMC-771 PANC-1	2.9 μM 29.3 μM 29.7 μM	Wu et al. (2013b)
Eupenicillium sp.	*Xanthium sibiricum*/roots	**12.** Eupenicinicol D	THP-1	8 μM	Li et al. (2017)
Penicillium sp.	*Tripterygium wilfordii*/ leaves	**13.** Funicone	KB	13.2 μM	Chen et al. (2014)
Penicillium melinii	*Panax ginseng*/root	**14.** Ginsenocin	MKN45 LOVO A549 MDA-MB-435 HepG2 HL-60	7.3 μM 8.4 μM 19.3 μM 5.3 μM 9 μM 1.9 μM	Zheng et al. (2013)
Massrison sp.	*Rehmannia glutinosa*/roots	**15.** Massarigenin D	L-O2 HepG-2 MCF-7 A549	84.8 μM 92.7 μM 49.9 μM 50.8 μM	Sun et al. (2011)
Penicillium brefeldianum	*Pinellia ternata*/rizhome	**16.** N-demethyl melearoride A	HepG2 U2-OS MDA-MB-231	> 50 μM > 50 μM 36.6 μM	Gao et al. (2017)
Phomopsis sp.	*Musa acuminata* /leaves	**17.** Oblongolides Z	KB BC NCI-H187 Vero	37 μM 26 μM 32 μM 60 μM	Bunyapaiboonsri et al. (2010)

(continued)

Table 11.3 (continued)

Fungal endophytic taxa	Medicinal plant/tissue	Compound isolated	Biological activity	IC_{50}	Reference
Cochliobolus kusanoi	*Nerium oleander*	**18.** Oosporein	A549	21 μM	Alurappa et al. (2014)
Massrison sp.	*Rehmannia glutinosa*/roots	**19.** Paecilospirone	L-O2 HepG2 MCF-7 A549	24.4 μM 20.4 μM 14.9 μM 13.4 μM	Sun et al. (2011)
Penicillium sp.	*Tripterygium wilfordii*/leaves	**20.** Penifupyrone	KB	4.7 μM	Chen et al. (2014)
Perenniporia tephropora	*Taxus chinensis var. maire*/bark	**21.** Perenniporin A	HeLa SMMC-771 PANC-1	108.2 μM 161.7 μM 157.2 μM	Wu et al. (2013b)
Phomopsis amygdale	*Corylus avellana*/roots, branches and leaves	**22.** Pestalotin	MDA-MB-231 PC-3 HT-29	194.6 μM 95.9 μM 77.9 μM	Akay et al. (2014)
Cryptosporiopsis sp.	*Clidemia hirta*	**23.** (*R*)-5-hydroxy-2-methylchroman-4-one	HL-60	25.9 μM	Zilla et al. (2013)
Phomopsis amygdale	*Corylus avellana*	**24.** (S)-4-butoxy-6-((S)-1- hydroxypentyl)-5,6-dihydro-2H-pyran-2-one	MDA-MB-231 PC-3 HT-29	94.6 μM 516.2 μM 320.6 μM	Akay et al. (2014)
Cephalotheca faveolata	*Eugenia jambolona*/leaves	**25.** Sclerotiorin	HCT-116	0.6 μM	Giridharan et al. (2012)
Massrison sp.	*Rehmannia glutinosa*/roots	**26.** Spiromassaritone	L-O2 HepG2 MCF-7 A-549	32.1 μM 25 μM 30.3 μM 43.7 μM	Sun et al. (2011)
Penicillium brefeldianum	*Pinellia ternata*/rizhome	**27.** Spirotryprostatin F	HepG2 U2-OS MDA-MB-231	35.5 μM > 50 μM 14.1 μM	Gao et al. (2017)

screened endophytic fungi associated with *Ricinus communis* for its inhibitory activity on AChE. They found six active strains, and the best results were from the extract of the fungus *Alternaria* sp. with 78% of inhibition and an IC50 of 40 μg mL^{-1}. Na et al. (2016) isolated the fungus *Geomyces* sp. from the plant *Nerium indicum* that showed high inhibitory activity with an IC50 value of 5.2 μg mL^{-1} that might be related to substances derived from vincamine produced by this fungus. Chapla et al. (2014a) identified six fungal isolates with inhibitory AChE activity recovered from the medicinal plant *Michelia champaca*, with the species *C. gloeosporioides*, *Phomopsis stipata*, and *Xylaria* sp. showing the highest activity.

Wang et al. (2016b) investigated the medicinal plant *Huperzia serrata* from the Jinggang Mountain region (China) for the presence of endophytic fungi with acetylcholinesterase inhibitory activity. From the 247 strains isolated, 221 generated extracts with in vitro AChE inhibitory activity, with 4 of them, namely, *Coletotrichum* spp., *Ascomycota* spp., *Sarcosomataceae* spp., and *Dothideomycetes* spp. causing more than 80% inhibition. Dong et al. (2014) analyzed *H. serrata* from the Tianmu Mountains of Hangzhou (China) for endophytic fungi producing huperzine A (HupA), a substance produced by the plant itself and known for its high AChE inhibitory activity. They found that the fungus *Trichoderma* sp. seems to produce this substance, yielding an extract capable of inhibiting AChE by 81.9%. The fungi recorded for producing HupA and other potential substances are listed in the Table 11.4.

Table 11.4 Compounds with activity of AchE inhibition reported from endophytic fungi from medicinal plants.

Fungal endophyte taxa	Host plant/Tissue	Compouds isolated	IC_{50}	Reference
Chaetomium sp.	*Huperzia serrata*	**1.** 3β-hydroxy-5,9-epoxy-(22*E*,24*R*) -ergosta-7,22-dien-6-one ($C_{28}H_{42}O_3$)	–	Yu et al. (2016)
Shiraia sp.	*Huperzia serrata*/leaves	**2.** Huperzine A ($C_{15}H_{18}N_2O$)	–	Zhu et al. (2010)
Chaetomium globosum	*Panax notoginseng*/seed	**3.** Epicoccolide B ($C_{18}H_{14}O_8$) **4.** 3-Methoxyepicoccone	5.5 μM –	Li et al. (2016a)
Alternaria alternata	*Vinca rosea*/branches	**5.** Altenuene ($C_5H_{16}O_6$)	–	Bhagat et al. (2016)
Aspergillus versicolor	*Huperzia serrata*/leaves	**6.** Avertoxin B ($C_{28}H_{37}O_9$)	14.9 μM	Wang et al. (2016b)
Cladosporium cladosporioides	*Huperzia serrata*/leaves	**7.** Huperzine A ($C_{15}H_{18}N_2O$)	–	Zhang et al. (2011)
Aspergillus terreus	*Artemisia annua*/stems	**8.** 16α-hydroxy-5-N-acetylardeemin ($C_{28}H_{28}N_4O_4Na$)	58.3 μM	Ge et al. (2010)
Bipolaris sorokiniana	*Rhayza stricta*/leaves	**9.** Bipolarisenol ($C_{16}H_{13}O_6$)	223.1 mM	Khan et al. (2015)

11.7 Antioxidant Activity

Antioxidant substances protect cells from injury caused by free radicals produced by the natural metabolism during aerobic respiration (Yehye et al. 2015). These radicals have an important physiological role but may cause toxic effects leading to degenerative diseases like cancer and Alzheimer's disease (Kaul et al. 2012; Yehye et al. 2015). The antioxidant activity of endophytic fungi extracts might be related to the production of flavonoid and phenolic compounds, making them act as reducing agents and hydrogen donors due to their redox properties (Qiu et al. 2010; Khan et al. 2017). Besides the uses in the pharmaceutical industry, the potent activity found in the endophytic extracts can be used as a natural antioxidant in the food industry (Nath et al. 2012; Rana et al. 2018a, b; Yadav et al. 2017). The importance of exploring new sources of effective antioxidants is related to the low number of antioxidants approved for clinical applications (Kaul et al. 2012).

The compound 1.1-diphenyl-2-picrylhydrazyl (DPPH) is a stable free radical widely accepted as a tool to analyze the antioxidant ability of extracts. When a substance with antioxidant activity interacts with DPPH, it transfers electrons or hydrogen atoms neutralizing its free radical character and causing changes in its color (Naik et al. 2003). Using this method, Singh et al. (2016) found phenolic compounds with IC_{50} value of 22.5 μg mL^{-1} in the extract of the endophytic fungus *Cladosporium velox*, isolated from the medicinal plant *Tinospora cordifolia*.

Tejesvi et al. (2008) searched for antioxidant activity in endophytic *Pestalotiopsis* species associated with medicinal plants growing in southern India. They found three fungi with significant scavenging activity (over 80%): *Pestalotiopsis theae* (TA-37), isolated from the bark of the medicinal plant *Terminalia arjuna*, presenting an IC_{50} value of 14 μg mL^{-1}; *Pestalotiopsis* sp. 3 (TA-60), isolated from the root of *Terminalia arjuna* with an IC_{50} value of 25 μg mL^{-1}; and *Pestalotiopsis virgatula*, isolated from the bark of *Terminalia chebula* with an IC_{50} of 27 μg mL^{-1}.

Nath et al. (2012) found four endophytic fungi with antioxidant activity occurring in the medicinal plant *Emblica officinalis*. The fungus *Phomopsis* sp. isolated from the stem showed the most significant IC_{50} value of 17.4 μg mL^{-1}, a value comparable with that of ascorbic acid (15 μg mL^{-1}) used as positive control. In addition, the fungi identified as *Diaporthe* sp. and *Xylaria* sp., isolated from the root and stem of *Epacris* sp., were also considered active, with IC_{50} values in the range of 18.9 μg mL^{-1}–29.4 μg mL^{-1}. The same group studied the fungi *Cholletotrichum gloeosporoides*, *Penicillium* sp., and *Aspergillus awamori*, all isolated from the plant *Rauwolfia serpentina,* for their ability to produce antioxidant compounds showing that *A. awamori* was most effective with extract disclosing the highest scavenging activity in the DPPH test (Nath et al. 2013).

Khiralla et al. (2015) investigated five Sudanese medicinal plants for endophytic fungi with potential antioxidant activity. Among 21 endophytes isolated, the fungus *Aspergillus* sp. from the seed of *Trigonella foenum-graecium* showed the most significant results, with an IC_{50} value of 18.0 μg mL^{-1} in the DPPH assay. Jayanthi

et al. (2011) reported that a *Phomopsis* sp. isolated from the medicinal plant *Mesua ferrea* disclosed an IC_{50} value of 31.3 μg mL^{-1}, while the positive control, ascorbic acid, showed an IC_{50} value of 11.1 μg mL^{-1}.

Shukla et al. (2012) showed that *Paecilomyces variotti*, one of the endophytic fungi isolated from the root of *Ocimum sanctum,* yielded an extract with IC50 value of 71.8 μg mL^{-1} in the DPPH test and 110.9 μg mL^{-1} for the scavenging of the hydroxyl radical. Yadav et al. (2014) disclosed the antioxidant activity and total phenolic content (TPC) of endophytic fungi isolated from *Eugenia jambolana.* They found two potential fungi with scavenging activity higher than 80%, *Chaetomium* sp. that present the highest concentration of phenolic compounds among all isolates and *Aspergillus* sp. Other two techniques were used to measure the antioxidant activity of these fungi: hydrogen peroxide scavenging assay and reducing power assay, confirming the antioxidant potential of compounds produced by these fungi.

Bhagobaty and Joshi (2012) isolated endophytic fungi from plants growing in the "sacred forests" of India. They measured their antioxidant potential using DPPH and FRAP assays. The latter measures the UV absorbance of ferrous ions. The tests showed that the fungus *Mortierella hyalina,* isolated from the plant *Osbeckia stellata,* has a good potential, with a FRAP value of 1.316 μM and a percentage of free radical scavenging activity of 79.7%. In these assays, the control substance ascorbic acid has a FRAP value of 2.000 μM and free radical scavenging activity of 64%.

Huang et al. (2007) isolated bioactive fungi from the medicinal plant *Nerium oleander* and used the ABTS method to test the total antioxidant capacity of the fungi extracts. Most of the fungal strains (75%) showed moderate antioxidant capacities with values ranging from 20 to 50 μmol trolox/100 mL culture. The fungus *Chaetomium* sp. presented the highest antioxidant capacity, that is, 151 μmol trolox/100 mL culture.

Srinivasan et al. (2010) evaluated the antioxidant property of the endophytic fungus *Phyllosticta* sp. isolated from the leaves of *Guazuma tomentosa* using the DPPH and ABTS methods. The results showed the potential antioxidant of the fungus extract, that contains phenolic and flavonoid substances, with EC50 values of 580 μg mL^{-1} for the DPPH radical test and 2030 μg mL^{-1} for the ABTS radical test.

Qiu et al. (2010) identified two flavonoid-producing endophytic fungi with antioxidant activity in the twigs of *G. biloba*. *Aspergillus nidulans* and *Aspergillus oryzae* showed antioxidant activity on the hydroxyl radical scavenging activity test of 34% and 58%, respectively. Substances from endophytic fungi isolated from medicinal plants that present antioxidant activity are listed in the Table 11.5.

11.8 Neglected Tropical Diseases

Neglected tropical diseases (NTDs) are a diverse group of infectious diseases caused by bacteria, parasites, protozoans, or viruses, which prevail especially in tropical and subtropical regions (Lenzi et al. 2018). According to reports published by World

Table 11.5 Compounds with antioxidant activity reported from endophytic fungi from medicinal plants

Fungal endophyte	Host plant/tissue	Compounds isolated	IC_{50} (DPPH)	Reference
Pseudocercospora sp.	*Elaeocarpus sylvestris*/stems	**1.** Terreic acid ($C_7H_6O_4$)	58.6 mM	Pirihantini and Tachibana (2017)
Fusarium solani *Fusarium oxysporum* *Fusarium proliferatum*	*Cajanus cajan*/roots	**2.** Cajaninstilbene acid ($C_{21}H_{22}O_4$)	-	Zhao et al. (2012b)
Cephalosporium sp.	*Trachelospermum jasminoides* /leaves	**3.** Graphislactone A ($C_{16}H_{14}O_6$)	9.6 mM	Selim et al. (2014)
Chaetomium globosum	*Panax notoginseng* /seeds	**4.** Flavipin ($C_9H_8O_5$) **5.** Epicoccone ($C_9H_8O_5$) **6.** 3-Methoxyepicoccone ($C_{10}H_9O_6$) **7.** Epicoccolide A ($C_{18}H_{14}O_9$) **8.** Epicoccolide B ($C_{18}H_{14}O_8$)	18.9 mM 58.6 mM 49.7 mM 13.9 mM 32.4 mM	Li et al. (2016a)

Health Organization (WHO), the diseases of major concern are Chagas disease and visceral leishmaniasis (WHO 2017).

The frequency of drug-resistant parasites has greatly increased, and most treatments involve highly toxic drugs. In addition, the chemotherapeutic agents used in patients with these diseases have lacked effectiveness. Thus, there is an urgent need to search for novel drugs from previously unexplored sources, including natural products, to combat the global health problems posed by parasitic infections (Martínez-Luis et al. 2011).

Historically, natural products are a good strategy when searching for new bioactive compounds, they provide a basis for both design and synthesis of derivative compounds aiming at optimizing biological activity and minimizing side effects (Scotti et al. 2010; Schulze et al. 2015). The ongoing development of new antiparasitic agents is important to overcome the limitations related to the high toxicity of the drugs currently available for the treatment of diseases caused by tropical parasites (Croft et al. 2006). Despite advances in the discovery and development of plant-derived drugs, NTDs continue to cause morbidity and mortality in hundreds of millions of people, especially in poor areas (Goupil and McKerrow 2014).

While endophytic fungi are an abundant and reliable source of metabolites with medicinal and agrochemical applications, they have been only scarcely explored as sources of antiparasitic agents (Martínez-Luis et al. 2011). Because these fungal endophytes are promising sources of bioactive metabolites, they could be used to produce important antiparasitic compounds to treat NTDs such as trypanosomiasis, leishmaniasis, and malaria.

11.8.1 Trypanosomiasis

Chagas disease (or American trypanosomiasis) is a parasitic illness that results from infection by the hemoflagellate protozoan *Trypanosoma cruzi* (*T. cruzi*). The transmission of Chagas disease occurs primarily through the bite of an infected triatomine bug on an individual. Triatomines are insects that usually belong to the genera *Triatoma*, *Rhodnius*, or *Panstrongylus*, which are commonly known as "barbeiros" in Brazil and "kissing bugs" in the United States, due to their preference for biting the faces of sleeping people. These insect genera include more than 140 species, of which 61 are endemic to Brazil (Costa and Peterson 2012). According to WHO, and in common with other neglected tropical diseases, "Chagas disease is a proxy for poverty and disadvantage: it affects populations with low visibility and little political voice, causes stigma and discrimination, is relatively neglected by researchers, and has a considerable impact on morbidity and mortality" (Coura and Dias 2009).

Approximately 7–eight million individuals have Chagas disease, and 50,000 new cases are diagnosed every year in Latin America, North America, and Europe. It is estimated that more than 90 million individuals are currently at risk of infection with the Chagas disease's etiologic agent (Coura and Dias 2009; WHO 2014; Vazquez et al. 2015). The conventional treatment is based on benzimidazole (Bayer Health Care—Lampit®) and nifurtimox (Roche— Rochagan® or Radanil®), which were developed over 100 years ago. Both drugs have strong side effects, such as appetite loss, vomiting, polyneuropathy, and dermopathy. The long-term treatment required combined with the strong side effects contributes to frequent desistence (Guedes et al. 2011). Additionally, benzimidazole and nifurtimox are mostly effective for the blood forms in the acute phase and not so effective against the intracellular forms in the chronic phase (Muelas-Serrano et al. 2002).

Human African trypanosomiasis (or sleeping sickness) is a fatal vector-borne parasitic disease caused by *Trypanosoma brucei brucei* transmitted by the tsetse fly (*Glossina* spp.). This neglected tropical disease occurs only in rural areas of sub-Saharan Africa (Simarro et al. 2011). To date, only a few drugs have been approved for the treatment of human African trypanosomiasis. These include suramin, pentamidine, melarsoprol, eflornithine, and the combination of nifurtomox/eflornithine. Most of the drugs are old, having been discovered in the 1940s and 1950s, and have adverse effects such as nausea, vomiting, fatigue, seizures, fever, diarrhea, hypoglycemia, abdominal cramping, peripheral neuropathy, hypertension, heart damage, and neutropenia on the patients (Jacobs et al. 2011). For the reasons describe above, mining and developing new trypanosomiasis drugs from natural products is crucial and essential because endophytic fungi offer a high number of natural products with diverse chemical structures and novel pharmacological mechanism of action.

11.8.2 Leishmaniasis

Leishmaniasis is a group of human diseases caused by protozoan species of the genus *Leishmania*, which are prevalent in tropical and subtropical areas of the world. Brazil is among the ten countries affected by 90% of the cases worldwide of both cutaneous and visceral leishmaniasis (WHO 2010). More than one million people are being victimized by leishmaniasis worldwide, and reported fatalities are of around 30,000 annually (Kamhawi 2017). There are around 20 species of *Leishmania* (*Trypanosomatidae*), which can cause three variations of the leishmaniasis disease: cutaneous, mucocutaneous, or visceral leishmaniasis (Dawit et al. 2013).

Leishmania (*Viannia*) *braziliensis* is the main etiological agent of American tegumentary leishmaniasis and has the highest incidence in Brazil. This group of infectious diseases has different clinical forms that are associated with the molecular diversity of the parasite and host immune response (Pereira et al. 2017). The visceral manifestation of the disease is usually caused by *Leishmania donovani* and *Leishmania infantum*, and it can affect internal body organs. It is also popularly known as kala-azar and can be fatal (Clem 2010).

There is no vaccine to control these diseases (Dawit et al. 2013). The current therapy consists of sodium stibugluconate (Pentosam®), meglumine antimonate (Glucantime®), miltefosine, amphotericin B, and paromomycin. The first drugs used for treatment were the antimonials. However, in the 1970s, the parasites started to show resistance to pentavalent sodium antimony gluconate, even at high doses, and as a result, these drugs were mostly abandoned. Miltefosine has replaced antimonials as a treatment in cases of resistance. However, it has also been associated with increasing resistance. Treatment with amphotericin B is effective, but it has highly nephrotoxic effects. The treatment can also be inhibited by cost, access, and difficulties in obtaining oral formulations of the drug (Hefnawy et al. 2017). Thus, there is a need for the discovery of new leads or scaffolds that can be used to develop less toxic drugs and alternative oral treatments (Prates et al. 2017).

11.8.3 Trypanocidal and Leishmanicidal Compounds from Endophytic Fungi

The major bioactive metabolites obtained from endophytic fungi associated with medicinal plants presenting trypanocidal and leishmanicidal activities are listed in Table 11.6. The fungi obtained from the medicinal plant *Caesalpinia echinata*, popularly known as Brazilwood, were tested against *L. amazonensis* and *T. cruzi*. The isolates from *Fusarium* sp., *Nectria mauritiicola*, and *Xylaria* sp. were able to inhibit *L. amazonensis* growth, and the isolate from *Fusarium sp*. was able to inhibit *T. cruzi* growth. The ethyl acetate (EtOAc) of *Fusarium* sp. showed the most promising result by inhibiting 92% of *T. cruzi* growth at a dose of 20 μg mL^{-1}. The extract of *Fusarium* sp. was subjected to fractionation, which revealed beauvericin as the

Table 11.6 Trypanocidal and leishmanicidal compounds reported from endophytic fungi associated with medicinal plants

Fungal endophyte	Medicinal plant/tissue	Compounds isolated	Biological activity	IC_{50}	Reference
Fusarium sp.	*Caesalpinia echinata*/stem	**1.** Beauvericin ($C_{45}H_{55}N_3O_9$)	*Trypanosoma cruzi*	1.9 μg ml^{-1}	Campos et al. (2015)
Nectria pseudotrichia	*Caesalpinia echinata*/stem	**2.** EtOAc extract	*Leishmania* (*Leishmania*) *amazonensis*	4.6 μg ml^{-1}	Campos et al. (2015)
Nectria pseudotrichia	*Caesalpinia echinata*/stem	**3.** 10-acetyl trichoderonic acid A **4.** 6′-acetoxy-piliformic acid **7.** Hydroheptelidic acid	*Leishmania* (*Viannia*) *brasiliensis*	21.4 μM 28.3 μM 24.8 μM	Cota et al. (2018)
Microthyriaceae sp.	*Paspalum conjugatum*	**10.** Sterigmatocystin ($C_{18}H_{12}O_6$)	*Trypanosoma cruzi*	0.1 μmol L^{-1}	Almeida et al. (2014)
Lasiodiplodia theobromae	*Vitex pinnata*/leaves	**12.** Cladosporim B **13.** Desmethyl-lasiodiplodin	*Trypanosoma brucei brucei*	17.8 μM 22.5 μM	Kamal et al. (2016)
Diaporthe phaseolorum-92C	*Combretum lanceolatum*/roots	**15.** 18-des-hydroxy cytochalasin H	*Leishmania* (*Leishmania*) *amazonensis*	9.2 μg ml-1	Brissow et al. (2018)
Aspergillus terreus	*Carthamus lanatus*/roots	**16.** Terrenolide S **17.** (22*E*,24*R*)-stigmasta-5,7,22-trien-3-β-ol **18.** Stigmast-4-ene-3-one	*Leishmania donovani*	27. 3 μM 15.3 μM 11.2 μM	Elkhayata et al. (2015)
Aspergillus terreus-F7	*Hyptis suaveolens*	**23.** Terrein ($C_8H_{10}O_3$) **24.** Butyrolactone I ($C_{24}H_{24}O_7$) **25.** Butyrolactone V ($C_{24}H_{24}O_8$)	*Leishmania* (*Leishmania*) *amazonensis*	23.7 μM 26.0 μM 78.6 μM	Silva et al. (2017c)
Aspergillus calidoustus	*Acanthospermum australe*/leaves	**26.** Ophiobolin K **27.** 6-epi-ophiobolin K ($C_{25}H_{36}O_3$)	*Trypanosoma cruzi*	13.0 μM 9.6 μM	Carvalho et al. (2015)
Cochliobolus sativus	*Vernonia polyanthes*/leaves	**28.** Mixture of cochlioquinone A and isocochlioquinone A **29.** Anhydrocochlioquinone	*Leishmania* (*Leishmania*) *amazonensis*	10.2 μg ml^{-1} 50.5 μg ml^{-1}	Nascimento et al. (2015)

EtOAc ethyl acetate

active compound. While the crude extract of *Fusarium* sp. showed an IC_{50} of 30 μg mL^{-1} (**2**) in the assay with *T. cruzi* forms expressing the β-galactosidase gene, beauvericin showed an IC_{50} value times smaller (1.9 μg mL^{-1}, 2.4 μM) (**1**). The EtOAc extract from the culture of *Nectria pseudotrichia* was active against amastigote-like forms of *Leishmania* (*Leishmania*) *amazonensis* showing an IC_{50} value of 4.6 μg mL^{-1} (**2**) (Campos et al. 2015). Fractionation of *Nectria pseudotrichia* extracts yielded seven compounds, 10-acetyl trichoderonic acid A (**3**), 6′-acetoxypiliformic acid (**4**), 5′,6′-dehydropiliformic acid (**5**), piliformic acid (**6**), hydroheptelidic acid (**7**), xylaric acid D (**8**), and cytochalasin D (**9**). Compounds **3**, **4**, and **7** were the most active against *Leishmania* (*Viannia*) *braziliensis*, with IC_{50} values of 21.4, 28.3, and 24.8 μM, respectively, and showed low toxicity to Vero and THP-1 cells (Cota et al. 2018).

When screening for natural products with antiparasitic activity, the endophytic fungus, *Microthyriaceae* sp., was isolated from aboveground tissue of the tropical medicinal grass *Paspalum conjugatum* (*Poaceae*) in Panama. Cultivation followed by bioassay-guided chromatographic fractionation of the extract led to the isolation of the new polyketide integrasone B (**9**) and two known mycotoxins, sterigmatocystin (**10**) and secosterigmatocystin (**11**). Sterigmatocystin was found to be the main antiparasitic compound in the extract of fermentation broth of this fungus, possessing potent and selective antiparasitic activity against *T. cruzi*, with an IC_{50} value of 0.13 μmol L^{-1}. Compounds **10** and **11** showed high cytotoxicity against Vero cells (IC_{50} of 0.1 and 1 μmol L^{-1} respectively) (Almeida et al. 2014).

The endophyte *Lasiodiplodia theobromae* obtained from the leaves of *Vitex pinnata*, a medicinal plant of Malaysia, displayed activity against *Trypanosoma brucei brucei*. Three known compounds were isolated, namely, cladospirone B (**12**), desmethyl-lasiodiplodin (**13**), and *R*-(−)-mellein (**14**). Cladospirone B and desmethyllasiodiplodin compounds exhibited good activity against *T. b. brucei* with minimum inhibitory concentrations of 17.8 μM and 22.5 μM, respectively (Kamal et al. 2016).

Brissow et al. (2018) demonstrated that crude EtOAc extracts of *Diaporthe phaseolorum*, an endophytic fungus isolated from the roots of *Combretum lanceolatum* Pohl ex Eichler, a Brazilian medicinal plant, showed trypanocidal activity at 20 μg mL^{-1}, reducing 82% of the number of amastigotes and trypomastigotes of *T. cruzi*. The compound 18-des-hydroxy Cytochalasin H (**15**) was isolated and evaluated for leishmanicidal and tripanocidal activities. The compound reduced the viability of *L. amazonenses* promastigotes with an IC_{50} value of 9.2 μg mL^{-1}.

From the endophytic fungus *Aspergillus terreus* isolated from roots of *Carthamus lanatus* L. (*Asteraceae*), one new butenolide derivative, Terrenolide S (**16**), together with six known compounds, (22E,24R)-stigmasta-5,7,22-trien-3-β-ol (**17**), stigmast-4-ene-3-one (**18**), stigmasta-4,6,8(14),22-tetraen-3-one (**19**), terretonin A (**20**), terretonin (**21**), and butyrolactone VI (22), has been isolated. Compounds **16**, **17,** and **18** exhibited antileishmanial activity toward *L. donovani* with IC_{50} values of 27.3, 15.3, and 11.2 μM, respectively, and IC_{90} values of 167, 40.6, and 14.7 μM, respectively (Elkhayata et al. 2015). The same kind of endophyte, the fungus *Aspergillus terreus* obtained from *Hyptis suaveolens* (L.) Poit, growing in the Brazilian wetland known as the Pantanal, showed trypanocidal and leishmanicidal

activities. Three compounds were isolated from the acetate extract of the fungal culture: terrein (**23**), butyrolactone I (**24**), and butyrolactone V (**25**). Compounds **23**, **24**, and **25** exerted moderate leishmanicidal activity against *L. amazonensis*, IC_{50} = 23.7, 26.0, and 78.6 μM, respectively. Furthermore, compounds **24** and **25** were examined for the trypanocidal effect on L929 cells from mouse connective tissue infected with *T. cruzi* amastigotes and promastigotes. Both compounds were inactive or toxic. Compounds **24** and **25** killed 100% of the cells at 94.2 and 181.6 μM, respectively. It was the first report on the leishmanicidal activity of compounds **23**, **24**, and **25** against *L. amazonensis* (Silva et al. 2017c).

Carvalho et al. (2015) obtained the endophytic fungus *Aspergillus calidoustus* isolated from leaves of *Acanthospermum australe* (*Asteraceae*), a medicinal plant native to the Brazilian savannah. From this endophyte, they recovered two compounds, ophiobolin K (**26**) and 6-epi-ophiobolin K (**27**), which showed trypanocidal activities with IC_{50} values of 13.0 and 9.6 μM against *T. cruzi*. However, these compounds were also cytotoxic to the fibroblast host cells of *T. cruzi*.

Nascimento et al. (2015) reported that endophytes associated with the medicinal plant *Vernonia polyanthes* are a potential source of leishmanicidal compounds. They recovered 16 endophythes from leaves of this plant growing in Brazil, and the fungal culture crude ethanol extracts were tested for their antileishmanial activity. The most active extract was obtained from *Cochliobolus sativus* (IC_{50} = 3.0 μg mL^{-1}). From this extract, a mixture of cochlioquinone A and isocochlioquinone A (**28**), and anhydrocochlioquinone A (**29**), was obtained. The mixture **28** exhibited a good antileishmanial activity, with an IC_{50} value of 10.2 μg mL^{-1}. Anhydrocochlioquinone A also presented an antileishmanial activity, but its IC_{50} value was five times higher (50.5 μg mL^{-1}).

11.9 Conclusion

Considering the high number of vegetal species living in the world, it is important to understand the methods and criteria to select the host plant for the study of endophyte communities in order to provide the best opportunities to isolate novel and potential endophytic fungi. Among the criteria used and described at the literature stands out the choice of medicinal plants (plants that have an ethnobotanical history), because that plants might be considered important reservoir of a promising source of novel endophytes and their compounds can be useful for human health and veterinary. The infectious/parasitic diseases and cancer, for example, discussed in this chapter still demand a special attention and need of investment in research considering the high mortality rate generated by some of them, together with the inexistence of an effective treatment without side effects and resistance. In this context, endophytic fungi are an alternative that might offer a high number of natural products with diverse chemical structures and novel pharmacological action's mechanism. Endophytic taxa mainly of the genus *Aspergillus*, *Chaetomium*, *Diaporthe*/*Phomopsis* complex, *Fusarium*, and *Penicillium* are potential producers

of bioactive compounds for the treatment of those diseases. Additionally, endophytes may contribute to their host plant and for the industry by producing a plethora of substances; however, the search for better treatments remains an important challenge and a constant niche to be explored.

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Chapter 12
Extracellular Carbohydrate-Active Enzymes of *Trichoderma* and Their Role in the Bioconversion of Non-edible Biomass to Biofuel

Vivek Sharma and Richa Salwan

12.1 Introduction

Constant depletion of fossil fuels, increasing world population and concerns for environments, in particular the impact of climate change on our ecosystem, demand futuristic sustainable technologies. India is presently ranked third in oil consumption. Moreover, growing population size, growth in automobile and other industrial sectors in India, led to increase in energy consumption. The need for environmental friendly and renewable energy resources such as biofuels produced from agricultural-based biomass can decrease our dependence on fossil fuels (Borin et al. 2017). Therefore, efforts for developing alternate energy resources are on high priority. As per the records of US Department of Energy, United States and Brazil contributed to approximately 80% (24,570 million gallons) of the global ethanol production (http://www.afdc.energy.gov) (Borin et al. 2017). The bioprospection of agricultural biomass in particular from non-edible sources can be a better alternative and sustainable approach with minimal environmental concerns in the future (Gaurav et al. 2017). Agricultural biomass which is often a major source for environmental pollution can be of vital importance for biofuel production as an alternate energy resource (Ning et al. 2016; Wan et al. 2001; Chirino-Valle et al. 2016). Limitations of biomass from grain-producing crops demand alternative second-generation biofuels from non-edible agricultural crops (Ayrinhac et al. 2011) and other biomass sources. These carbohydrates from different non-edible biomass can be explored for biofuel using a combination of enzymes (Gaurav et al. 2017). Biofuels are categorized into three generations on the basis of raw material. Initially for first generation

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biofuel, crops plants were explored followed by second generation agricultural by-products and marine resources such as seaweeds and cyanobacteria (Demirbas 2008; Kang et al. 2014; Gaurav et al. 2017).

Biofuels from second-generation agricultural wastes offer several benefits. Biofuels from renewable resources can be exploited as promising and almost carbon-neutral fuel enhancers of octane in unleaded gasoline for cleaner combustion which can reduce environmental pollution. Plant biomass containing lignocellulose in terrestrial ecosystems is one of the most potential raw materials due to its availability, price and high sugar content (Barros-Rios et al. 2016; Zhao et al. 2016). The basic constituents of lignocellulose include cellulose, hemicellulose and lignin (Sindhu et al. 2016) which are interconnected through covalent and non-covalent bonds (Gaurav et al. 2017; Zhang et al. 2017). Cellulose which is a major part of plant biomass has been widely recognized and explored for developing sustainable processes and can help in mitigating the impact of climate change, occurs through consumption of fossil fuels (Gupta and Verma 2015; Zhang et al. 2017). Conversion of lignocellulose-based plant biomass is a major bottleneck in developing sustainable processes for alternate energy resources and other value-addition products (Kuhad et al. 2011; Villares et al. 2017). The breakdown of recalcitrance lignocellulose and chitin containing biomass using chemical pretreatment often results in toxic side effects to the ecosystem (Margeot et al. 2009; Wang et al. 2017) (Fig. 12.1).

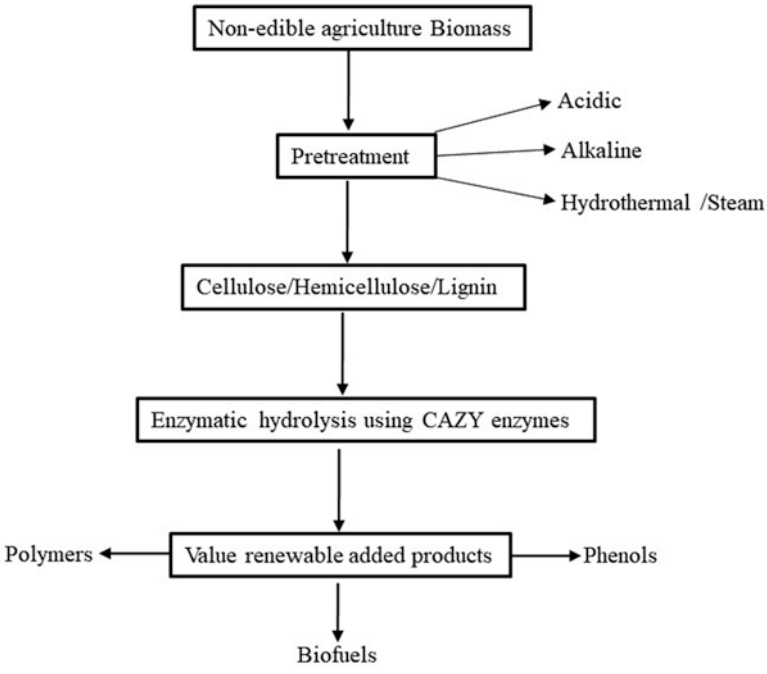

Fig. 12.1 A schematic overview of the conversion of non-edible agricultural biomass to value-added products using carbohydrate-active enzymes

The conversion of plant biomass into value added products can be achieved through the breakdown of recalcitrant plant biomass via pretreatment and enzymatic hydrolysis (Zhang et al. 2017). Efficient utilization of the lignin, hemicellulose and cellulose can decrease the cost of biofuel production up to 25% (Zhao and Xia 2009, 2010; Zhao et al. 2018a).

Enzymes in the CAZy database are categorized into four classes: glycoside hydrolases (GHs), polysaccharide lyases, glycosyl transferases and carbohydrate esterases. The glycosyl hydrolases (GHs) have potential to break the non-edible biomass into oligo- or monomers (Ferreira Filho et al. 2017). Additionally, a family of auxiliary enzymes known as lytic polysaccharide mono-oxygenases (LPMOs) which is a major component of saprophytic fungi like *Trichoderma* and *Aspergillus* catalyses copper-dependent oxidation of C-H bonds in complex polysaccharides (Obeng et al. 2017; Borin et al. 2017; Monclaro and Filho 2017; Cologna et al. 2018) (Fig. 12.2).

In general, the stains of *Trichoderma* are used as biocontrol agents due to their diverse attributes (Sharma and Shanmugam 2012; Sharma et al. 2013, 2016a, b, 2017a, b, 2018a, b). Besides this, enzymes from filamentous fungi such as *T. reesei* are paradigms for industrial application in paper, textile, pulp, food and biofuel processing industries (Kumar et al. 2008; Singhania et al. 2010; Seiboth et al. 2011; Marx et al. 2013; Tiwari et al. 2013) (Table 12.1).

Laccases or phenol oxidases and lytic mono-oxygenases can enhance the activity of lignocellulases and thus lower the enzyme required to break down alkali-pretreated

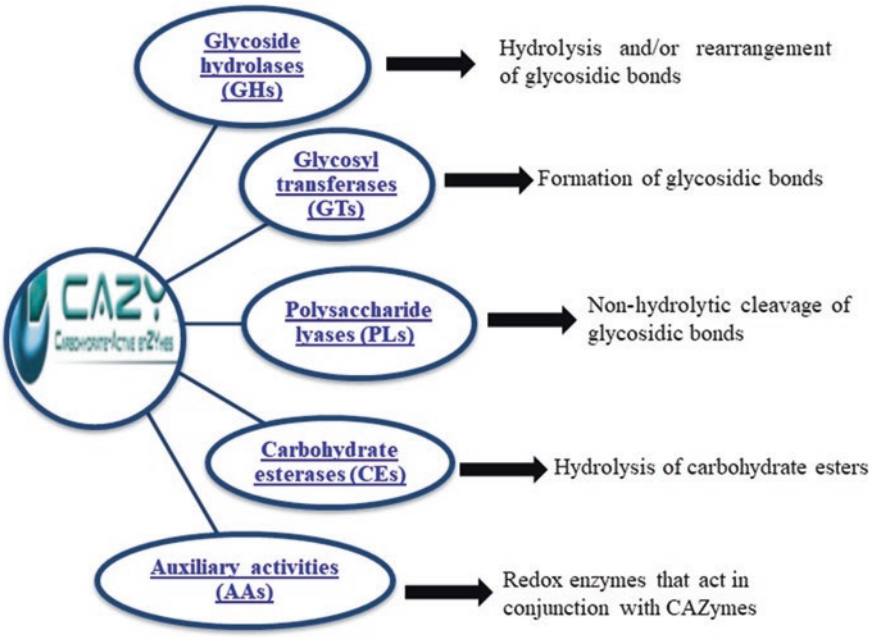

Fig. 12.2 Overview of carbohydrate-active enyzmes as per cazy database http://www.cazy.org/

Table 12.1 Recent examples of CAZymes for different applications

Enzymes/CAZy family	Fungal species	Role	References
Cellulase complex	*T. reesei* SCB18	High cellulase capacity for biomass saccharification and high β-glucosidase (BGL) activity	Gao et al. (2017)
Glycosyl hydrolases such as GH1, GH3, GH18, GH35 and GH55 families of chitinases, glucosidases, galactosidases, and glucanases	*T. hamatum* strains YYH13 and YYH16	Offers scope for developing β-glucosidase with high cellobiose-hydrolysing efficiency	Cheng et al. (2017)
GH95, GH67, GH62, GH54, GH43, GH26, GH11 and GH10	*T. reesei* RUT-C30	Hemicellulose degradation	Ferreira Filho et al. (2017)
GH7, CBM1; GH5, GH7, GH12, GH45, GH1, GH3 and GH6 families; 23 CBM1 domains; two auxiliary families	*T. harzianum*, *T. reesei* RUT-C30	Cellulose degradation	Borin et al. (2017); Ferreira Filho et al. (2017)
Chitinase	*T. saturnisporum* and other *Trichoderma* species	Protoplast isolation, fungal pathogen management, treatment of chitinous waste	Dahiya et al. (2006); Sharma and Shanmugam (2012); Sharma et al. (2017a, b, c)
Glucanases	*T. harzianum*	Fungal pathogen suppression through mycoparasitism	Sharma et al. (2017a, b, c)
Cellobiohydrolase I	*T. longibrachiatum*	The action of LPMOS promoted the efficacy of cellobiohydrolase I, endoglucanase and β-glucosidase in pretreated bacterial microcrystalline cellulose	Song et al. (2018)
Lytic polysaccharide mono-oxygenase	*T. reesei*		
Lytic polysaccharide monooxygenase, AA9 and CBM1	*T. reesei* RUT-C30	Acts on cellulose and β-glucan	Borin et al. (2017)
Xylan esterase, CE3, CE5 and CBM1	*T. reesei* RUT-C30	Acts on xylan	Borin et al. (2017)
Xylanase, GH10 and GH11	*T. reesei* RUT-C30	Acts on xylan	Borin et al. (2017)
Xylanase	*T. reesei* QM6a	High biotechnological relevance	Ramoni et al. (2017)

agricultural biomass containing lignocellulose (Ladeira Azar et al. 2018). For examples, xylanase of *A. niger* and *T. reesei* are found to be inhibited by the presence of phenol at 1.5 mg and 0.3 mg per mg of protein, respectively. On the other hand, laccases of *C. cubensis* and *Penicillium pinophilum* are reported active at a concentration of 35 mg of phenol per mg of protein (Ladeira Azar et al. 2018). The glcyosyl hydrolase family plays a vital role in the breakdown of complex plant biomass, whereas the role of the auxiliary activity (AA) family has been discussed in recent studies (Levasseur et al. 2013). Among different CAZymes, β-1,4/(1,3)/(1,6)-type glycosyl hydrolase family breaks down complex plant polysaccharides to oligomers or monomers (Vu and Marletta 2016). Lytic mono-oxygenases belonging to AA9 (formerly GH-61), AA10 (formerly CBM-33) and AA11 enzymes are capable of targeting recalcitrant non-edible carbohydrates such as chitin, cellulose, starch and other polysaccharides containing β-linkages between glucose and substituted glucose units (Ravalason et al. 2012; Vu et al. 2014; Gong et al. 2015; Ning et al. 2016). The genomes of *A. niger* and *T. reesei* share about the same (2.5%) proportion of CAZymes in comparison to total predicted genes; still, the transcriptomic response of *A. niger* is found to be diverse and revealed upregulation of 190 CAZymes which belong to 62 different families, whereas for *T. reesei*, 105 CAZymes belonging to 51 families were upregulated (Borin et al. 2017).

The recent developments in genomic, transcriptomic, metabolomic or proteomic technologies have led to the identification of several CAZymes and other genes of *Trichoderma* which are active during agricultural biomass degradation. Keeping in view the importance of CAZymes in plant biomass degradation for various applications, attempt has been in present chapter to provide an overview of different lytic enzymes of *Trichoderma* strains in white biotechnology for biofuel production.

12.2 Biocatalysis of Plant Biomass Using Lignocellulases

Lignocellulose from plant biomass is the major raw material for biofuels, foods and other livestock feeds (Kumar et al. 2008). Studies on fungal lignocelluloses-mediated lysis have revealed several pathways for lignin metabolism (Mansur et al. 2003). The lignocellulose is a promising biomass pretreatment alternative, and fungal lignocellulases are one of the potential enzymes in debasing lignin of plants (dos Santos et al. 2007; Dias et al. 2007; Plácido and Capareda 2015;Martinez et al. 2009). Moreover, the lignocellulases are also explored for the removal of toxic compounds as well as supplementing the pre-existing technologies of sugar hydrolysates after conventional pretreatment (Plácido and Capareda 2015; Bilal et al. 2018). Higher white fungi are known to produce a plethora of lytic enzymes. The lignin-degrading enzyme complex in white fungi is mainly consists of lignin peroxidase, manganese peroxidase and laccase along with other enzymes which include peroxidase, aryl alcohol oxidase, glyoxal oxidase and oxalate. The broad specificity of substrates also makes them vital enzymes which are capable of breaking a wide range of xenobiotics and pollutants having structural similarities to lignin (Hofrichter

2002; Bilal et al. 2018). A combination of co-culture techniques is found to enhance production of these enzymes. For example, 2.6-fold enhancement in laccase activity compared to *C. comatus* monoculture with higher delignification of up to 66.5% and conversion of 82% of polysaccharides into fermentable sugars was recorded (Ma and Ruan 2015).

12.2.1 Cellulases

Cellulase, a complex of three enzymes, leads to the complete breakdown of cellulose to glucose units which can be used as fermentable sugar for biofuel production. The cellulose is degraded initially through endoglucanase (EG) (1,4-β- D-glucan-4-glucano-hydrolases) (EC 3.2.1.74) by random action into oligomers which are then targeted by exoglucanase (EC 3.2.1.74 and EC3.2.1.91) into cellobiose and glucose units. The β-glucosidases belonging to EC 3.2.1.21 hydrolyse the cellodextrins, cellobiose into glucose units (Keshwani and Cheng 2009; Jeya et al. 2009). The cellobiohydrolases (CBHs, named as CBH1 and CBH2), β-glucosidases (BGLs) and endoglucanases (EGs) act in a coordinated and complementary fashion to hydrolyse cellulose (Cavaco-Paulo et al. 1997; Gusakov et al. 2007; Jørgensen et al. 2007; Ma et al. 2011). The cocktail of different cellulolytic enzymes play vital role in the hydrolysis of complex plant polysaccharides. For example, a mixture of CBH1, CBH2 and EG1 is found to responsible for up to 80% of cellulose breakdown (Rosgaard et al. 2007). *T. reesei*, an industrial strain, is known to secrete CBH1, CBH2, EG1, EG2, EG3 and EG5 which act in a synergistic manner to completely hydrolyse the lignocellulose (Fang and Xia 2013). CBH1 and CBH2 are reported as major components of cellulase complex and accounts for 50–60% and 10–15% of the secreted protein, respectively (Rosgaard et al. 2007). Compared to CBH1, the specificity of CBH2 is approximately twice for crystalline cellulose (Zhou et al. 2008), and optimum synergism is reported at a 2:1 molar ratio (Zhou et al. 2009).

The other components of cellulase complex in *T. reesei* such as endo-β-1,4-D-glucanases are reported from glycosyl hydrolase families GH5, GH7, GH12 and GH45, whereas cellobiohydrolases are reported from families GH6 and GH7. The GH7 family contains endo-β-1,4-D-glucanases of CEL7B, previously known as EGL1 and CBHs (CEL7A, named as CBH1). The family GH5 cellulases is mostly explored from fungi strains (Li and Walton 2017), and three candidates of this family have been reported from *T. reesei*. The enzymes of GH7 family are distributed commonly. The orthologues of CEL7A cellulases are prevalent in the secretome of fungi-degrading biomass. The members of GH6 family comprise cellulase which acts exclusively from the non-reducing end of cellulose chain. The synergistic action of CEL7A and CEL6A is considered to play a key role in biomass degradation. The members of GH12 are typically low molecular weight (25 kDa) and do not contain cellulose-binding domain (CBM1) and glycosylation site. Due to their small size, GH12 can diffuse deeper into cellulosic material, and hence preferred for their role in laundry industry. On the other hand, members of GH45 cellulases are in

general small and have a wide substrate range compared to families GH5 and GH7. The members of GH45 enzymes share interesting structural similarities to plant expansins. Further intensive research efforts with genetic engineering strategies for single-enzyme cellulase components have increased the scope of *T. reesei* strain's improvement (Pryor and Nahar 2015; Qian et al. 2016, 2017; Wang and Xia 2011; Zhang et al. 2010).

12.2.2 β-Glucosidase

A heterogeneous family containing exo-glycosyl hydrolases catalyses the cleavage of β-glycosidic bonds in disaccharide or glucose-substituted molecules (Bhatia et al. 2002; Chandra et al. 2013; Cheng et al. 2017; Leah et al. 1995; Zagrobelny et al. 2008). According to the classification of CAZy (http://www.cazy.org) (Henrissat 1991; Cantarel et al. 2009), β-glucosidases are classified into two families: 1 and 3 of glycosyl hydrolases (Jeng et al. 2011). These enzymes enhance the action of cellulose-degrading enzymes by releasing phenolic compounds and hence are an attractive choice for renewable bioenergy. β-glucosidases hydrolyse the oligosaccharides and cellobiose oligomeric units obtained after the endoglucanases and cellobiohydrolases activities into monomeric glucose (Chandra et al. 2013).

The β-glucosidases of *T. reesei* are categorized into GH1 and GH3. The members belonging to family GH1 are exclusively intracellular in nature, whereas GH3 β-glucosidases are predominantly extracellular (Guo et al. 2016). CEL3A previously categorized as BGL1 is responsible for majority of the β-glucosidase activity. The 'exo/endo' concept revealed that CEL7A is also able to act in endo-manner; therefore, it is not a true exocellulase (Stahlberg et al. 1993; Kurasin and Valjamae 2011). However, neither the EGs nor the CBHs from fungi can cause massive cellulose decomposition (Payne et al. 2015). The lytic polysaccharide mono-oxygenases which were identified previously as endoglucanases belonging to GH61 (Sharma et al. 2018b) are now known as auxiliary family and cleave β-glucan in an oxidative fashion. The members of the family GH61 are also reported for their weak endoglucanase activity. The genome of *T. reesei* (http://www.genome.jgipsf.org/Trire2/Trire2.home.html) is reported to contain at least 10, β-glucosidases-encoded genes which include cel1A, cel1B, cel3A, cel3B, cel3C, cel3D, cel3E, cel3F, cel3G and cel3H. The gene encoding cel3A (bgl1) was found to be major extracellular β-glucosidase, whereas cel1A (bgl2) (Saloheimo et al. 2002a, b) and cel1B (Zhou et al. 2012) were reported to be intracellular. Additionally, cel3B, cel3E, cel3F, cel3G and cel3H are assumed to be extracellular, and cel3C, cel3D and cel3H are depicted as intracellular (Guo et al. 2016). Different knockouts, amino acid substitution and mutation of the BglR transcription factor in the PC-3-7 strain have been used to reveal the function of β-glucosidases (Fowler and Brown 1992; Zhou et al. 2012; Nitta et al. 2012; Xu et al. 2014; de Porciuncula et al. 2013; Shida et al. 2015; Li et al. 2016).

12.2.3 Xylanases

With a backbone of β-(1-4)-linked xylose units, polysaccharide xylan are structurally diverse and complex polysaccharides and predominantly composed of hemicelluloses which are linked to cellulose microfibrils (Scheller and Ulvskov 2010). The side chains are connected through C2 and C3 positions of D-xylosyl units (Puls and Schuseil 1993), and these chains can be substituted with acetyl, 4-methyl-D-glucuronosyl or L-arabinosyl units (Wong et al. 1988; Dodd and Cann 2009). Endo-β-1,4-xylanases or β-1,4-D-xylan xylanohydrolases (EC 3.2.1.8) are one of the important lytic components which can target the glycoside bonds in xylan backbone internally (Biely 1985; Polizeli et al. 2005; Mangan et al. 2017). Members of xylanase family belong to glycoside hydrolase (GH) families 5–12, 16, 26, 30, 43, 44, 51 and 62. Enzymes classified in 16, 51 and 62 families contain two catalytic domains compared to 5–11 and 43 families which have a true catalytic domain with endo-1,4-β-xylanase activity. The 9, 12, 26, 30 and 44 families may possess residual or secondary xylanase activity.

In recent classifications based on hydrophobic cluster analysis of catalytic domains and amino acid sequence similarities, xylanases are classified as GH10 and 11 and have a retaining type of mechanism. The information on catalytic properties of families 5, 7, 8 and 43 are very limited. The members of GH families 5, 7, 8, 10, 11 and 43 are different in their structure, mode of action, physicochemical properties and substrate specificities (Collins et al. 2005). The members of GH 10 family include high-molecular-mass proteins with cellulose-binding and catalytic domains and are connected through linker peptides. The estimated pI is 8–9.5 with $(\alpha/\beta)_8$ fold TIM barrel structure. On the other side, the GH11 family with low molecular mass and pI are further divided into two, alkaline and acidic (Buchert et al. 1995; Juturu and Wu 2012). The GH11 members exclusively catalyse endo-β-1,4–mediated cleavage (EC 3.2.1.8) in xylan and hence are also known as true xylanases. The high catalytic efficiencies of these enzymes due to small size, vast temperature and pH optima provide them an edge for their exploitation in various biotechnological applications (Paes et al. 2012).

Xylanases of *Trichoderma* are one of the widely explored enzymes, and Rut C-30 strain of *T. reesei* is well explored for commercial applications of xylanase and cellulase production (Gerber et al. 1997). The xylanases produced by *T. harzianum*, *T. lignorum*, *T. koningii*, *T. longibrachiatum*, *T. pseudokoningii* and *T. viride* also have been investigated (Silveira et al. 1999; Chen et al. 2009). Xylanases from a psychrotrophic *Trichoderma* strain have been characterized (Zhou et al. 2011) and genes encoding them have been cloned from *Trichoderma* species and expressed in heterologous hosts such as *E. coli* (Min et al. 2002), *S. cerevisiae* (Ahmed et al. 2005) and *P. pastoris* (He et al. 2009). In the *T. reesei* genome, three xylanases belonging to the GH11 family have been identified, and two of these were reported in the early 1990s, whereas the third GH11 xylanase XYN5 was identified in a recent study (Martinez et al. 2008; Dos Santos Castro et al. 2014; Peciulyte et al. 2014; Saloheimo and Pakula 2012).

12.2.4 Lytic Polysaccharide Mono-oxygenases (LPMOs)

The recently discovered enzyme class, the LPMOs, stimulates the hydrolysis of plant biomass and enhances the efficacy of glycosyl hydrolases (Hu et al. 2014). Unlike cellulases which target glycosidic bonds by hydrolysis, LPMOs are copper dependent and catalyse the breakdown of polysaccharides through oxidation at C1 or C4 glucose units in the presence of external electron donors.

12.2.5 Laccases

Laccases are also known as phenol oxidases or benzenediol: oxygen oxidoreductase (EC 1.10.3.2) belongs to the multicopper oxidase (MCO) family and represents a group of metalloenzymes. These enzymes are used in various biotechnological applications. The search for strains producing such laccases has gained increased attention in recent times. In general, laccases are monomeric glycoproteins of 60–70 kDa in size, and carbohydrates approximately contribute to 30% of their molecular weight (Cázares-García et al. 2013). Laccases oxidize compounds containing a variety of phenolic, diamines and aromatic amines (Abd El Monssef et al. 2016). In lignocelluloses containing biomass, laccases play an important role in developing a clean biocatalytic process and improve cellulose recovery from feedstocks containing lignocellulose (Avanthi and Banerjee 2016). Additionally, the affinity of laccases for different aromatic compounds make them a promising and attractive tool for de-colouration and detoxification of different synthetic dyes and phenolic pollutants. These chemicals are often a source of water contamination and thus can cause problems to public health and our environment (Anbia and Ghaffari 2011). A combination of laccases and cellulases enhances delignification and thus increases the efficiency of developing enzymatic processes for biofuels and other value-added product generations such as coal solubilization (Chakroun et al. 2010). The extracellular laccase of *T. virens* is reported for their role in mycoparasitism against the sclerotia of plant pathogens such as *Botrytis cinerea* and *Sclerotinia sclerotiorum* (Catalano et al. 2011; Cázares-García et al. 2013).

Fungi of basidiomycetes and ascomycetes division are known to degrade lignin, xenobiotics, chemicals used for guaiacol synthesis and vanillin metabolites at industrial scales (Dekker et al. 2002; Halaburgi et al. 2011; Younes and Sayadi 2011). The wood-rotting fungi such as *Trametes* spp., *Cerrena maxima*, *Lentinus tigrinus*, *Coriolopsis polyzona* and *Pleurotus eryngii* are prominent laccase producers (Saloheimo and Niku-Paavola 1991; Morozova et al. 2007; Madhavi and Lele 2009). In general, fungal laccases are known to possess high redox -potential and broad substrate specificity compared to laccases of bacterial origin. The pH optima of fungal laccases is reported at acidic pH, whereas for bacterial laccases, like oxidases, it operates close to neutral-alkaline pH (Kolomytseva et al. 2017). Laccases can target phenolic constituents of lignin and have compatibility to work at industrial

pH, in solvents and at especially high temperatures and therefore are potential source for wood delignification for bioethanol production (Shanmugam et al. 2018). The laccase-encoding genes from other fungi such as *Pycnoporus sanguineus* and *Phlebia radiata* have been cloned and expressed using the Pcbh1 promoter and the Tcbh1 terminator of *T. reesei* (Zhao et al. 2018b).

Among ascomycetes, *Trichoderma* species have been extensively explored for cellulase production (Tsao and Chiang 1983). *Trichoderma* strains with laccase activity are more efficient in breaking natural substrates than strains without these enzymes (Assavanig et al. 1992). The laccases from ascomycetes have characteristic features which are not present in basidiomycetes laccases. The presence of additional L1–L4 signature domains (Kumar et al. 2003) helps their differentiation from other multicopper oxidases. The laccase activity has been reported in strains of *T. atroviride*, *T. reesei*, *T. viride*, *T. longibrachiatum* and *T. virens* (Assavanig et al. 1992; Krastanov et al. 2007; Gochev and Krastanov 2007; Catalano et al. 2011; Cázares-García et al. 2013). In addition, the conidia of *T. atroviride*, *T. viride* and *T. harzianum* are also reported for laccase activity (Holker et al. 2002; Pokorny et al. 2005). Studies on purification and characterization of laccases of extracellular nature have been conducted in *T. harzianum* (Sadhasivam et al. 2009), *T. atroviride* (Chakroun et al. 2010) and *T. reesei* (Levasseur et al. 2010) strains. The infection in *Pleurotus ostreatus* cultures with *T. viride* spores is also reported to induce higher laccase activity (Divya et al. 2013).

12.3 Distribution and Identification of CAZy Genes in *Trichoderma* Genome

The comprehensive information on carbohydrate-active enzymes glycoside hydrolases, carbohydrate esterases, polysaccharide lyases and glycosyltransferases which contribute to the breakdown and modification of glycosidic bonds can be gained from CAZy database. A number of enzymes and active transcripts involved in plant biomass degradation have been identified using genomics, transcriptomics or proteomics approaches. The majority of these transcripts have been identified as glycosyl hydrolases and carbohydrate esterases (Fig. 12.3).

In industrial strain *T. reesei*, a limited number of carbohydrate active enzymes (CAZymes) have been characterized, whereas genome sequencing revealed presence of several candidates genes which may have been transferred horizontally from bacteria (Häkkinen et al. 2012). Phylogenetic analysis of different CAZy genes has identified around 201 glycoside hydrolase-encoding genes, 22 carbohydrate-encoding esterase genes and 5 polysaccharide lyase genes. Among glycosyl hydrolases, β-glucosidases of GH3, α-galactosidases of GH27 and chitinases of GH18 have been reported in abundance (Häkkinen et al. 2012). In the genome of *T. reesei*, 61 CAZy families were predicted which exclude family CE10. The complete list of CAZy families in *T. reesei* can be obtained from a study

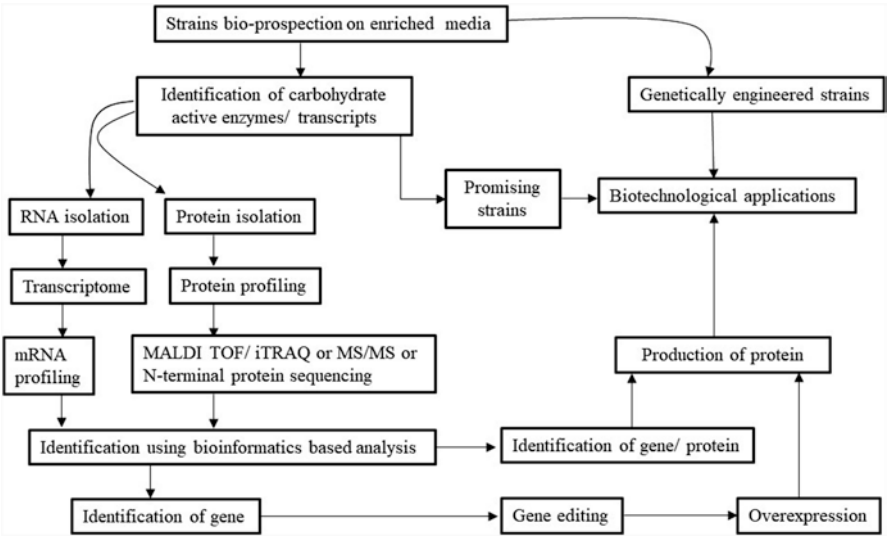

Fig. 12.3 An overview of mining carbohydrate-active candidate transcripts/proteins for biomass conversion

conducted by Häkkinen et al. (2012). A comparison overview of *T. reesei* CAZY enzymes with other fungi revealed that cluster containing AGLIII and other four candidate α-galactosidases are restricted to *T. reesei*. The cluster for β-glucuronidase genes of families GH79, GH18 and GH92 also revealed expansion in *T. reesei*, whereas families GH43 and Gh61 showed reduction (Häkkinen et al. 2012). The CBHI/CEL7A and CBHII/CEL6A acts in exo-fashion on cellobiohydrolases whereas five endo-acting cellulases such as EGII/CEL5A, EGI/ CEL7B, EGIII/ CEL12A, EGV/CEL45A and EGIV/CEL61A are also reported form *T. reesei* strain (Penttilä et al. 1986; Saloheimo et al. 1994; Saloheimo et al. 1997; Okada et al. 1998). Additionally three putative endoglucanases (CEL74A, CEL61B and CEL5B) were reported (Foreman et al. 2003). In the genome of *T. reesei*, two β-glucosidases (BGLI/CEL3A and BGLII/ CEL1A) (Barnett et al. 1991; Fowler and Brown 1992; Takashima et al. 1999; Saloheimo et al. 2002a, b) and five β-glucosidases (CEL3B, CEL3D, CEL1B, CEL3C, CEL3E) also have been reported (Foreman et al. 2003). A protein named as swollenin (SWOI) involved in the biomass degradation by disrupting cellulose crystalline structure without the release of sugars has been also reported (Häkkinen et al. 2012). On the other hand, a number of other enzymes such as xylanases (XYNI, XYNII, XYNIII and XYNIV), mannanase (MANI) (Stalbrand et al. 1995), acetyl xylan esterase (Foreman et al. 2003; Margolles-Clark et al. 1996a), α-glucuronidase (GLRI) (Margolles-Clark et al. 1996a), arabinofuranosidases (ABFII and ABFIII) (Margolles-Clark et al. 1996b; Foreman et al. 2003; Herpoël-Gimbert et al. 2008), α-galactosidases (AGLI, AGLII and AGLIII) (Margolles-Clark et al. 1996b; Zeilinger et al. 1993) and β-xylosidase (BXLI) (Margolles-Clark et al. 1996c, d) has also been reported from *T. reesei* and other

filamentoru fungi (Tenkanen et al. 1992; Torronen et al. 1992; Xu et al. 1998; Knob et al. 2010). These proteins are known to play a vital role in breaking xylan-derived oligosaccharides. Also, several novel candidate lignocellulose-degrading genes have been identified from *T. reesei* genome (Martinez et al. 2008).

Screening of *T. harzianum* isolate for CAZymes via RNA-Seq and bioinformatics approach revealed around 259 transcripts related to glycoside hydrolases, 101 transcripts for glycosyl transferases, 6 for polysaccharide lyases, 22 for carbohydrate esterases, 42 for auxiliary activities (AAs) and 46 for carbohydrate-binding proteins when cellulose was used as substrate. The highest number of genes has been reported from GH18, GH3, GH16, GH2 and GH5 families. For hemicellulases, 24 glycosyl hydrolases belonging to families GH10, GH11, GH26, GH43, GH54, GH62, GH67 and GH95 were identified. The maximum enzymes were reported from GH43 and GH95 families, whereas the lowest number was identified from GH67, GH62, GH54, GH26 and GH10 families (Ferreira Filho et al. 2017).

12.4 Strain Improvements

Strains of *T. reesei* have been the topic of investigation for its cellulases. Higher enzyme production cost is one of the key hurdles involved in commercial applications of these enzymes for biofuel production. Screening for high level of cellulase-producing strains is an efficient strategy to address this issue. Due to high porduction cost of enzymes, efforts are required to enhance the production, intrinsic activity and reinforcing the existing biomass degrading enzymes with auxiliary proteins (Wilson 2009; Horn et al. 2012; Peterson and Nevalainen 2012; Hu et al. 2015; Müller et al. 2015; Payne et al. 2015). A number of tools such as genetic engineering advance genetic transformation based on use of marker or marker-free selections, or RNA interference has been discussed by Bischof and Seiboth (2014). *T. reesei* Rut-C30 and *T. reesei* D-7 mutants developed by the use of basic chemicals such as ethyl methyl sulfonate (EMS), and other methods such as plasma irradiation are already used for high cellulase production. The filter paper activity and corn starch hydrolysate higher cellulase production in *T. reesei* strain D-7. Mutant-based study has been successful in obtaining potential cellulase-producing mutants (Zhang et al. 2017). In the last decades, efforts on strain improvement using traditional mutagenesis and screening methods have resulted in *T. reesei* strains RUT-C30 capable of producing up to 30 g/l of extracellular cellulases (Eveleigh and Montenecourt 1979; Eveleigh 1982) and even producing as high as 100 g/l of extracellular protein (Cherry and Fidantsef 2003). The commercial formulation for enhanced cellulase production such as Novozymes and Dupont are also obtained through mutations in *T. reesei*. In recent studies, the advancements of molecular tools in gene/genome engineering using specific insertion or deletion or mutation of nucleotides have been explored to meet the growing demands of different

biomolecules including enzymes. The discovery of Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) and CRISPR-associated (cas) 9 genes (CRISPR/cas9) system has democratized the genome engineering in a flexible manner either at a single- or multi-loci-based genome-wide modification. The CRISPR/cas9 system nowadays has emerged as a powerful tool for strain improvement in filamentous fungi such as *T. reesei* (Liu et al. 2015; Donohoue et al. 2018).

12.5 Conclusion and Future Prospects

The exploration of microbe's innate capacity to convert complex polysaccharides into biofuels with octane value is one of the predominant research areas presently. The filamentous fungi such as *T. reesei* have been widely extensively for cellulase and hemicellulase production. The genetic manipulation of *T. reesei* using mutagenesis has led to improved strains with higher cellulase production. Advancements in biotechnological tools have significantly contributed in developing alternate and efficient technologies. Enzymatic treatment offers advantage over chemical and physical methods being environmentally friendly. In several studies, either single or a combination of physical and chemical methods of mutations such as UV irradiation, ethyl methanesulfonate and N-Methyl-N′-nitro-N-nitrosoguanidine had been deployed in *Trichoderma*, *Aspergillus* and other fungi. The commercial formulation developed by the enzyme industry in companies such as Novozymes and Dupont was obtained through mutations for enhanced cellulase production in *T. reesei*.

Enzyme-mediated delignification has been used for enhancing enzyme production using rational, semi-rational and directed evolution-based molecular and protein engineering strategies. In rational approach, modification through the use of site direct mutagenesis for lignolytic enzymes such as laccases has been used successfully. Alternatively, a mixture of two filamentous fungi such as *T.reesei* and *A. niger* has been found better for cellulase production. Despite the challenge associated with the expression of active recombinant proteins in heterologous system, paucity of signal peptides and expression system, genetic engineering through the use of codon optimization and substitution with unnatural amino acids in recombinant proteins is emerging field and can provide us enzyme systems with better catalytic property and enhanced self-life. However, the concern for low or lack of production of potent hemicellulases and β-glucosidases in *T. reesei* secretome needs alternative potential strategies which could either replace or supplement *T. reesei* enzyme system. Therefore, efforts are required for exploring microbial enzymes for biofuel production from agricultural biomass.

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Chapter 13
Fungal Biofuels: Innovative Approaches

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13.1 Introduction

Biofuel is a sustainable source of energy as it has been an option for fossil fuels that cause environmental destruction (Nithya Devi and Velayutham 2011). Such concern has been focused in order to preserve our valuable resources from depletion and illustrate the need for sustainable development. Biofuels can be obtained from various sources such as vegetable oil, seeds, lignocellulose, animal fat feedstocks, microbes, etc. Biofuels can be diverse in nature such as biodiesel, bioethanol, biohydrogen, cellulosic ethanol, etc. There are numerous advantages associated with biofuels like less global warming as it develops less carbon dioxide in comparison with fossil fuels. Generation of biofuel from microorganism is helpful in minimizing the waste and putting it into best use. Fungus supports in the degradation of biomass and additionally allows the conversion of agricultural waste to biofuel. Biomass processing techniques are divided into two categories: biochemical conversion and thermo-chemical conversion. Biochemical conversion leads to production of bioethanol and biodiesel. Various endophytic fungi grown and cultured on potato dextrose agar (Strobel 2014) have been explored that produce compounds such as alkanes, cyclohexanes, cyclopentanes, and alkyl alcohols/ketones, benzenes, and polyaromatic hydrocarbons that are found in biodiesel. As liquid fuel

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obtained from fossil fuels is depleting due to enormous consumption, alternative source is being explored. The growth of biofuel such as hydrocarbon has been a new method for examining these compounds for other applications.

Among different oleaginous microorganisms, numerous benefits have been observed to filamentous fungi such as the following: (1) it exhibits good lipid profile for generating high-quality biodiesel; (2) it generates oil with the help of solid state fermentation owing to less energy consumption; and (3) various carbon sources are used for the generation of lipid. White rot fungi have the potential to degrade cellulosic material and produce ethanol. Oleaginous fungi are isolated by the serial dilution method for the lipid generation, and lipid is extracted by Bligh and Tyer method. Lipids generated are utilized for physio-chemical properties for the production of biodiesel (Nithya Devi and Velayutham 2011). It has been revealed that biofuel generation is economical and eco-friendly because CO_2 emission is less. The methods involved in the production of ethanol involve separate hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SSF) (Fazeli et al. 2016). The enzyme is a protein that has a catalytic property that alters the rate of reaction. Cellulases and hemicellulases are the enzymes responsible for degrading cellulosic material, and *Trichoderma reesei* is involved in an enormous production of various cellulose and hemicellulose.

Nanotechnology has been a new approach, and it has enhanced the generation of biofuel. White rot fungi are highly effective, and it degrades lignin and is additionally preferred for fungal pretreatment (Cook et al. 2015). Various processing conditions play an important role in lignin degradation such as moisture, temperature and aeration is crucial for lignin degradation. Another strategy to solid state fermentation and direct enzyme approach is *in planta* generation of fungal enzymes in plants bound for biofuel yield. In this chapter, we have focused on the generation of fungal biofuel by various methodologies in order to conserve natural resources for sustainable development.

13.2 Classification of Biofuels

Biofuel has been generated in different parts of the countries, and it has been classified into different levels. The first biofuel produced was bioethanol, obtained by fermenting sugars. After its discovery, fuels were extracted from various sources such as vegetables or animal fats that are basically known as second-generation biofuels. Now latest innovations are made, and focus has been switched to generate biofuels from different microorganisms; this generation is regarded as third-generation biofuels.

13.2.1 First-Generation Biofuel

First-generation biofuels are biodiesel produced using oilseed crops, bioethanol established by fermenting sugars, extracted from different starch-loaded crops. Simple processing methods but with involvement of various fermentation organisms led to the generation of varieties of biofuels such as butanol and ethanol. Alternative source is searched to save our environment from CO_2 emission by the help of biofuel generation such bioethanol, butanol and the profile of bioethanol is analysed by aerated and non-aerated condition. The final efficiency is calculated using three factors: yield, productivity and final product concentration. Brazil is the largest producer of ethanol. Almost 40% of the fuel's uptake was developed in Brazil in 2005. There are many reasons associated with this: the unique feature of sugarcane as a product (sucrose); it is not a polysaccharide rather it is a disaccharide thus it does not necessitate processing (Elshahed 2010). Molasses is an agricultural waste, having high sugar content for bioethanol production-ranging sucrose (32%), fructose (16%) and glucose (14%). Batch fermentation technique was performed at an anaerobic and aerobic condition. The analytical methods, such as yeast cell count and total soluble solids of the fermentation broth, were analysed by direct counting procedure using total plate count and handheld refectrometer, respectively (Jayus et al. 2016).

13.2.2 Second-Generation Biofuel

Second generation biofuels are produced from biomass in another sustainable fashion, which is indeed carbon neutral or unchanging carbon damaging in the speech of its opinion on CO_2 concentrations, the duration 'plant biomass' refers for the most part to lignocellulosic cloth as this builds up the larger percentage of the second-rate and the plentiful nonfood equipment presented from plants (Gomez et al. 2008). However, biofuel extraction from agricultural by-products can lead to the rising need for liquid fuels. This has generated impressive advantage in manufacture of specific biomass crops as feedstock for biofuel assembly. Lignocellulosic equipment are an assortment of feedstocks for higher biofuels and can be obtained through hydrolysis and fermentation or through gasification. Lignocellulosic biomass is gasified to produce syngas, which in turn is transformed into DME (Balat 2006). The 2nd creation biofuels are comparatively immature consequences they be thought to declare usefulness capability for cost reductions and larger than before construction efficiency levels as supplementary event is carried place. It is then expected to befit a duty of the result to the challenge of shifting the joy sector towards other sustainable energy sources at a little scaffold in the medium-term. The possibility and sustainability of first-generation biofuels fabrication is

questionable looked-for to Second age bracket biofuels increases land-use transformation and land-use efficiency, and requires complicated handing out fabrication equipment (Stevens et al. 2004) on the core of existing scientific erudition and technology projections, third-generation biofuels specifically derivative from bacteria and microalgae are careful to be a viable other energy source devoid of the chief drawbacks such as food–fuel competition, land-use change, etc. (Nigam and Singh 2011).

13.2.3 Third-Generation Biofuel

Various efforts are being made to shift the use of fossil fuels to biofuels in order to save the environment from ecological imbalance. Hence, microbial species are being stressed in order to generate biofuels out of biodiesel, biohydrogen, bioethanol, etc. (Elshahed 2010). Cellulose is comprised of fine fibrils, lignin and hemicellulose, and it exhibits indissoluble properties despite heavy treatments. Lignin has always remained a barrier for cellulose extraction. Therefore, effective methods have been drawn in order to depolymerise lignin to benefit the biofuel industry. Lignin is depolymerised by releasing the enzyme lignin peroxidase with the assistance of white rot fungi. Lignocellulosic biomass is pretreated with white rot fungi prior to saccharification (Cook et al. 2015). Cellulose hydrolysis by various fungal enzymatic treatments improves sugar release and leads to the generation of biofuel products. Endophytic fungi produce volatile products, and the technique that monitors volatile organic compounds is called proton transfer reaction mass spectroscopy through fungal culture. Nuclear magnetic resonance method admonishes hydrocarbon generation by fungal culture (Strobel 2014). The output of hydrocarbon is duplicated as fungi are co-cultured in association with *E.coli*. Table 13.1 describes the advantages and disadvantages of third-generation biofuels. Further, third-generation biofuels that are being grown with the assistance of fungi will be discussed.

Table 13.1 Pros and cons of third-generation biofuels

Pros	Cons
They are renewable	More research work is needed
It can absorb carbon dioxide	Its production still needs a lot of work
Its basic sources grows fast like they need water, mushroom etc.	It needs higher amount of CO_2 to perform efficiently
It promises high content of energy	Contamination can be an issue
They do not need arable land	Challenges include feedstock production

13.3 Innovative Approaches for Fungal Biofuel Generation

There is tremendous improvisation in the approaches toward fungal biofuels, and the recent developments are testimonials. Diverse fungi strains have been envisaged for evaluating their biofuel efficiency. Biofuels derived from fungi include bioethanol, biodiesel, biohydrogen, and cellulosic ethanol. Different methodologies have been explored that led to the production of third-generation biofuels with the help of various fungi. Fig. 13.1 highlights biofuel from lignocellulosic biomass.

13.3.1 Bioethanol

Bioethanol is a renewable source of energy, and it is most often considered among all the fuels being generated from liquid fuels. Bioethanol has come as a substitute for the fuels that are confined, and its depletion is happening at a quicker rate. Recently, an organism has been distinguished that generates ethanol, and such strain is *Mucor indicus*. *Mucor indicus* is the fungus, can grow at aerobic and anaerobic conditions. There has been a great potential usage of fungal biomass for the

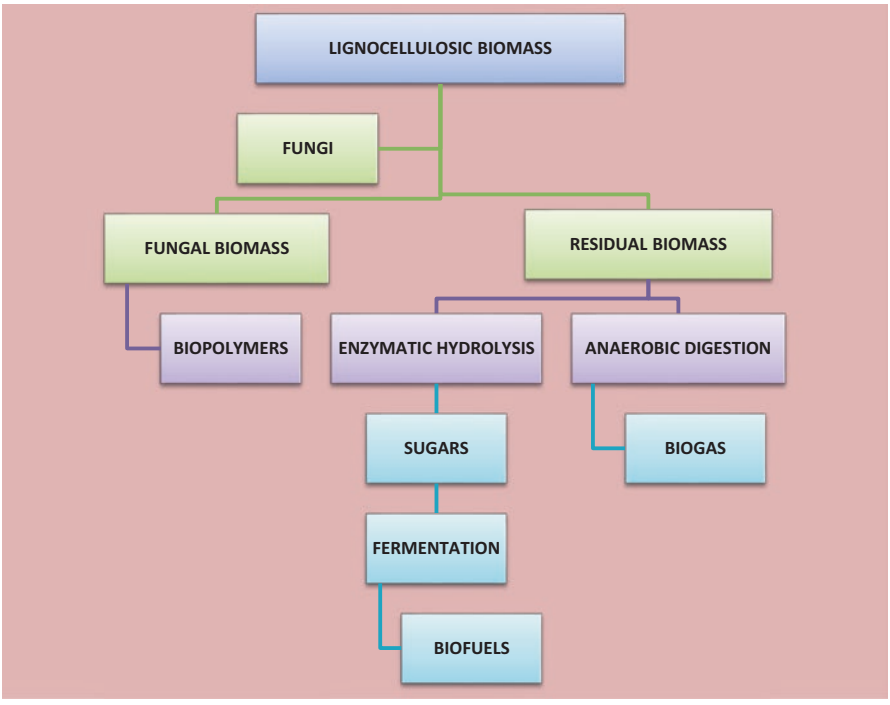

Fig. 13.1 Fungal biofuels from lignocellulosic biomass

worthful products, due to the structural importance of cell walls (Asachi et al. 2011). High cost has always remained an issue in many applications. The cost of yeast extract is high resulting in its restricted application in industrial operations. Therefore, it is necessary to produce a medium that satisfies the conditions required for microbial fermentation. Different enzymes are generated with the assistance of fungi such as *Trichoderma*, *Aspergillus*, *Monilia*, *Fusarium*, and *Rhizopus*. Diverse procedures are utilized in order to convert cellulose to ethanol, for instance, separate hydrolysis and fermentation (SHF), simultaneous saccharification and fermentation (SSF), direct microbial conversion (DMC), and consolidated bioprocessing (CBP) (Fazeli et al. 2016). Certain fungi such as *Monilia*, *Fusarium*, *Rhizopus*, *Aspergillus*, and *Neocallimastix* have the power to convert cellulose to ethanol. Table 13.2 illustrates the various microorganisms, substrates, and approaches involved for the generation of bioethanol.

It has been discovered that ethanol generation from rice straw has been focused by few technologies such as simultaneous saccharification and fermentation in association with *Mucor indicus, Rhizopus oryzae*, and *saccharomyces cerevisiae* and it was likened with cellulose and established that all the fungi were capable of generating ethanol from rice straw, with 40–74% production of the maximum theoretical SSF yield. *Rhizopus* is beneficial in performing fermentation in ethanol production.

Table 13.2 Substrates, microorganisms, and methods for bioethanol production

Substrate	Microorganisms	Methods	Biofuels	References
Rice Husk	*Aspergillus niger* *Aspergillus fumigatus*	Fermentation Enzyme Hydrolysis	Bioethanol	Ezeonu et al. (2014)
Horticultural Waste	*Trichoderma reesei* *Saccharomyces cerevisiae*	Solid-state fermentation	Bioethanol	Xin et al. (2013)
Tamarind Fruit	*Saccharomyces cerevisiae* (local strain) *Saccharomyces cerevisiae* MTCC 170	Stationary fermentation Shaking fermentation	Bioethanol	Ali and Kha (2014)
Banana Pseudo Stem	*Aspergillus ellipticus* *Aspergillus fumigatus* *Saccharomyces cerevisiae* NCIM 3570	Fermentation Enzymatic hydrolysis	Bioethanol	Ingale et al. (2016)
Alkaline pretreated sugarcane bagasse	*Phlebia* sp. MG-60	Consolidated bioprocessing (CBP)	Bioethanol	Maryana et al. (2014)
Waste Paper Sludge	*A. cellulolyticus*	Simultaneous saccharification and fermentation (*SSF*)	Bioethanol	Prasetyo and Park (2013)

Ethanol production occurs from the fungus identified from soil. *Aspergillus ochraceus* and *Saccharomyces cerevisiae* were utilized for saccharification and fermentation. Saccharification and fermentation occurs at stationary and shaking condition for a fermentive ethanol generation (Ali and Kha 2014). In order to optimize ethanol production, substrates were held at different concentrations such as 5 g substrate and 100 ml of distilled water; 5 g of substrate, 100 ml distilled water, and 0.5% glucose; and 5 g substrate and chemically defined medium. High output prevailed from sawdust supplied with a chemically defined medium in shaking fermentation. It has been observed that sawdust hydrolyzed by cellulases of *A. ochraceus* was most productive in terms of ethanol output and can therefore be harnessed in biofuel generation. Fig. 13.2 shows the different stages in the production of bioethanol.

Horticultural wastes can generate bioethanol through various technologies. Horticulture wastes have been estimated for cellulose generation practicing SSF by *T. reesei* (Xin et al. 2013). An integrated procedure is developed with the involvement of two technologies for the generation of ethanol fuel. The crude fungal enzyme complex produced from horticulture waste by the method, solid state fermentation and organosolv-pretreatment of horticultural waste for enzymatic cellulose saccharification, for diminishing sugars that can be matured to ethanol fuel (Xin et al. 2013). *Mucor elegans* fungi are vantages for second generation fuel producing ethanol in equivalence to the conventional ethanolic yeast. They have a tendency to ferment a variety of sugars to a range of useful products. Fungi generate enormous useful by-products such as microbial oil, protein, etc. There has been a novel approach for biofuel production and the transition from lignocellulosic biomass to sugars using anaerobic fungi such as *Pecoramyces ruminantium* strain C1A.

Biofuel generation from lignocellulosic biomass goes through inefficient saccharification. Thus, in order to have better efficiency, we need to infer the role of transcriptional ordinance and reactions of filamentous fungi to lignocellulose. Banana (*Musa acuminata*) helps in the accretion of cellulosic biomass wastes that serves as a novel material for ethanol generation. SSF has remained a remarkable technology for biofuel generation. Two fungal strains were used such as *A. fumigatus* and *A. ellipticus* on banana pseudo-stem by co-culture fermentation in order to expel reducing sugar, which was later utilized as a substrate for ethanol generation

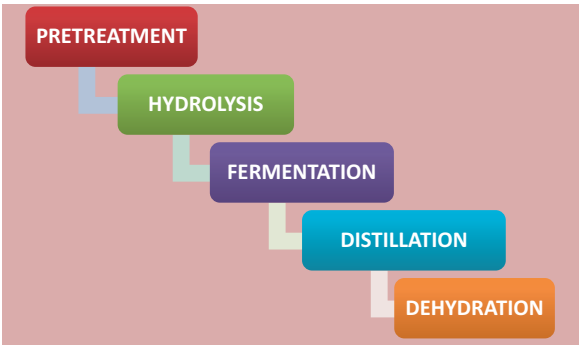

Fig. 13.2 Stages in Ethanol Production

by yeast strain such as *S. cerevisiae* NCIM 3570 (Ingale et al. 2016). Ethanol is also generated by the involvement of chitosan, contagious parasites of spineless creatures, for example, nematophagous growth, Pochonia *chlamydosporia* and the entomopathogenic fungi, *Metarhizium anisopliae*and *Beauveria bassiana*. *Pochonia chlamydosporia* is the biggest manufacturer of ethanol from chitosan (Aranda-Martinez et al. 2017).

The yield of the bioethanol age could be upgraded by *P. chlamydosporia* strain choice or hereditary building assurance for chemical advancement or brought articulation up keeping in mind the end goal to utilize this growth for ethanol creation at a major scale. The rise in biofuel revelation is for long haul managed exertion.

13.3.2 Biodiesel

In the modern society, petroleum-based fuels have been an important energy source. But because of an enhancement in demands for energy, the world is facing the danger of energy shortage due to decrease in the reserves of fossil fuels. Many governmental and industrial efforts are focusing on exploring for an alternative of petroleum-based fuels, and biodiesel which is obtained from microbes and specially fungi and can accumulate lipid is the best alternative. External carbon can metabolically transform into carbohydrates or hydrocarbon, and then they are changed into lipids, which are an essential storage compound. Microorganisms are regarded as oleaginous when the lipid amount in the cell exceeds 20% or further if their cell mass is made up of lipid biomass. Biodiesel is a renewable fuel which is compatible with the present-day commercial diesel, and it also helps in enhancing degradation (Vicente et al. 2004). Every fuel has some benefits and some drawbacks; in the case of biodiesel, one of the major drawbacks is high manufacturing cost. Hence, discovery of new products which can limit the cost of biodiesel is now on the rise. Microorganisms have the capability of producing biodiesel; they have some benefits over vegetable oil from oleaginous plants. Table 13.3 lists the lipid contents of some microorganisms for the generation of biodiesel.

Microorganisms do not need arable land and also gather high levels of lipids. Oleaginous species are referred to those microorganisms which gather more than 20–25% lipids (Ratledge 1991). These microbial lipids are used as raw materials for

Table 13.3 Lipid content of some microorganisms

Strains	Lipid content	Reference
Aspergillus oryzae	57	Meng et al. (2009)
Cunninghamella echinulata	40–47	Santos-Fo et al. (2011)
Mortierella isabellina	68–86	Meng et al. (2009)
Mucor circinelloides	20	Santos-Fo et al. (2011)
Mortierella vinacea	66	Meng et al. (2009)
Humicola lanuginose	75	Meng et al. (2009)

biodiesel production. *Mucor circinelloides* from fungi is used for the production of biodiesel. It has many features which favor its use for producing biodiesel, which include high amount of lipids, that is, around 25% dry mass (Ratledge 2002). During the submerged batch cultivation, they produce good biomass in bioreactors by utilizing a good range of carbon sources. The US Department of Energy (DOE) has chosen this fungus for its genome sequencing at the Joint Genome Institute after seeing its potential in producing biodiesel. The point of this article is to focus on utilization of biodiesel as a potential alternative to fossil fuel, which are used as a replacement of petroleum-based products. The fossil fuels will be over by the end of the century and the oleaginous fungi can optimize the condition for higher lipid production, which will help in the biodiesel production (Shatha 2017).

13.3.2.1 Methods for the Production of Biodiesel

There are two methods used in the production of biodiesel from *M. circinelloides*. The first one is the extraction of lipids from the biomass of *M. circinelloides* which are then transformed into FAMEs (fatty acid methyl esters), and the second method is the direct conversion of biomass from *M. circinelloides* without the need for previous extraction of lipids. Good-quality biodiesel is obtained with the help of the direct method. This then proposes that *M. circinelloides* biomass have the ability to be used as a feedstock for the production of biodiesel.

Lipids are extracted from a lyophilized and ground microbial biomass. Dry biomass of direct transformation was carried out through the technique introduced by Lewis et al. (2002). For the production of biodiesel, biomass of *M. circinelloides* was collected from the phototropic strain of MU241. Three mixtures of solvents are used for the lipid extraction and these are in the ratio of chloroform and methanol, chloroform, methanol, water and n-hexane. The n-hexane is used to prevent the usage of chlorinated solvents, because of their harmful effects on the environment. To replace chloroform, lots of solvents have been studied, and n-hexane came out as a better alternative for lipid extraction (Miao and Wu 2006). The production of biodiesel from *M. circinelloides* by direct alteration of fungal biomass without using any intermediate is a feasible method.

Biodiesel can also be produced from fungi before and after the exposure of UV light. *Aspergillus terreus*, the oleaginous fungal isolate used in this test, was exposed to UV light for 5 minutes and 10 minutes. To avoid photoreaction, the UV exposed spore suspension was kept in the dark. UV treated spore suspension and untreated spore suspension were injected into liquid medium, and after incubation, it was followed by extraction of biomass of selected oleaginous fungal isolates to estimate the dry weight and production of lipids. Thus, it is found that *A. terreus* fungal isolate can produce high biomass of dry weight to increase exposure to UV light (Shatha 2017).

This is probably because of the enhancement in the lipolytic enzyme activity which causes an increase in the accumulation of lipid intracellular cells. This makes

A. terreus a better alternative to producing biodiesel. UV radiation helps in the improvement of strains of fungi. Several attempts have been made for high production of biodiesel with the help of fungi. Table 13.4 enumerates the microorganisms and methods involved in production of biodiesel.

13.3.3 Biohydrogen

Most growths are oxygen consuming, yet anaerobic organisms has brought changes in freshwater lakes, landfill destinations, remote ocean dregs, and rumens of herbivores. Additionally they produce microbial products by the help of biogas reactors. These growths, keep up bounteously operational polysaccharide-corrupting compounds, fabricate them rousing for biomass debasement and assorted biotechnological applications. As a choice of mitochondria, anaerobic parasitic species participate in hydrogenosomes, organelles that control hydrogenase and convey sub-atomic hydrogen, carbon dioxide, acetic acid derivation, and distinctive blends as metabolic junk collect. The natural development of subatomic hydrogen in the rumen vivifies methanogenesis, and anaerobic organisms and methanogenic archaea are in a fundamental relationship that develops the metabolic emerged from the single-parasite structure (Magnus et al. 2016).

Anaerobic parasites are overpowering recognized from rumens of herbivores, some places they are inferring players in the corruption of lignocellulosic works twine. Anaerobic parasitic species get no mitochondria and are not capable to make imperativeness by any incredible or anaerobic breath. Rather, they contact their fundamental needs by the development of starches (general equation $C_xH_{2y}O_y$), a handle in which the imperativeness spring goes about as commonly the electron acceptor and the electron benefactor. As a substitute of mitochondria, anaerobic parasites get a handle on hydrogenosomes, organelles accomplished by coupling the

Table 13.4 Microorganisms and methods for biodiesel production

Microorganism	Method	References
Mucor circinelloides	Extraction of lipids from the biomass transformed into FAMEs and direct conversion	Gemma et al. (2009)
Aspergillus terrus	With the help of UV light	Shatha (2017)
Aspergillus sp.	With the help of direct (DTE) and indirect (IDTE) transesterification methods	Venkata Subhash and Venkata Mohan (2011)
Mucorfragilis	Disruption, oil extraction, and fermentation. Conditions were enhanced by response surface methodology	Huang et al. (2016)
Aspergillus oryzae Cunninghamella echinulata Mortierella isabellina	Acid catalyzed transesterification reactions with methanol producing methyl esters and then examined through chromatographic (GC-FID) and spectrometric techniques (MS, NMR ^1H)	Meng et al. (2009)

processing of glucose to cell essentialness age. Hydrogenosomes cover hydrogenase, and in the course of action with of spoiled lignocellulosic set fiber, they convey atomic hydrogen, carbon dioxide, acetic acid derivation, formate, lactate, and ethanol as metabolic revealed items (Magnus et al. 2016).

13.3.3.1 Biohydrogen by Endophytes

Most endophytes can be readily created to measure research facility media with potato dextrose stock being one of the best decisions. These creatures will by and large rise on area media completed with minimum salts, a nitrogen text style, and a couple of proper sugar polymers, for example, fiber or hemicellulose (Strobel 2014).

Various volatile compounds (VOCs) are delivered with the help of growths that prompt generation of biofuels. SPME (solid phase micro-extraction) is one of the most common method for the qualitative analysis of VOCs that is generated by fungi. A fairly new framework using proton turn over outcome pile spectroscopy PTR-MS has been effectively associated with execute, complete bona fide time observing of VOC making by contagious societies. Additionally, NMR (nuclear magnetic resonance) strategies have been adjusted to monitor hydrocarbon generation by fungi (Strobel 2014).

13.3.3.2 Anaerobic Fungi in the Biohydrogen Production Process

An ordinarily experienced issue amidst anaerobic absorption is constrained degradability of plant biomass: 40–60% of characteristic carbon stays unused (Procházka et al. 2012). This issue is an aftereffect of the physical structure and the unyielding manufacturing process of these polymers. In detail, lignin stays indigestive under anaerobic conditions and also shields cellulose, also hemicellulose from enzymatic degradation. Along these lines, progressions that can redesign anaerobic defilement of lignocellulosic biomass are required.

Presentation of an incomprehensible pre-treatment meander for plant improvement through for example white and brown rot fungi ruin creatures or the strong cellulose tainting Trichoderma viride has displayed promising results on redesiging the going with anaerobic retain limit in biogas reactors (Procházka et al. 2012; Wagner et al. 2013). On the other hand, anaerobic developments into these bioreactors would take out the need of an oxygen exhausting pre-absorption. Concerning the presented objectives, mesophilic conditions are qualified.

The probability of *Anaeromyces* and *Piromyces* strains to sort out into biogas-passing on anaerobic flood bacterial framework, to redesign corruption of substrate polysaccharides and along these lines to influence methane creation has as of late been endeavored in explore center conditions. Promising outcomes were gotten amidst the bioaugmentation of swine compost empowered biogas reactors with specific strains of anaerobic parasites. Modification to parasitic biomass incited 4–22%

higher gas yields and up to 2.5% higher methane fixation (Procházka et al. 2012; Fliegerová et al. 2012). An advancing considers demonstrating that bioaugmentation with anaerobic living creatures did not broaden the general, methane yield, regardless, it animates beginning gas age and in this way may decrease bolster time (Nkemka et al. 2015). By and large, in any case, it was unrealistic to save parasitic movement, and the contagious valuable impact on hydrolysis appears to decay after around ten long periods of brooding.

13.3.4 Cellulosic Ethanol

Cellulosic ethanol is ethanol generated biologically from a cellulosic total mass of living organism from a given area of land which is derived from grasses, algae, cultivable land and forestry residue and in some fast growing wood, which is a unique sustainable conveyance fuel that strengthen the economy, environment, and strategic attributes (Brethauer and Wyman 2010). The economic competition for producing cellulosic ethanol is determined by the feedstock cost, constituting about 35–50% of total ethanol production cost (Hess et al. 2007) cellulosic ethanol in further aspects, development of biofuel industry could lower the demand of gas drilling, oil and nuclear power. Cellulose and hemicellulose, when undergo chemical reaction can be adapted for new purposes into ethanol with a process of well-developed technologies (Zheng et al. 2009). Genetically modified plants fabricate cellulases and hemicellulases which lower the requirement for pretreatment process. Enzymatic breakdown of the high solids process is observed as the primary disadvantage affecting the ethanol yield. In wearing down of cellulosic ethanol, AFEX (ammonia fiber expansion) is pretreated, which is found in increasing metabolic yield and ethanol production (Lau and Dale 2009). Pretreatment of cellulosic substances is needed to achieve high yields despite the various methods of biotechnology which is cost-effective; pretreatment has major impacts in advancing the significant to reduce cost and hasten commercial implementation.

For the large scale production of ethanol fuel the cellulosic application (Farrell et al. 2006) will definitely be required and ethanol is adapted to do the need, research, and technology (Yang and Wyman 2007). Ethanol from corn grains and soybeans for biodiesel can be passed on for natural advantages potential vitality harvests and microorganism's ready to separate biomass is lethal for building up the possibilities of eloquent cellulosic biofuel production, with large quantities and lower food supply. Transportation biofuels, for example, synfuel hydrocarbons or cellulosic ethanol, when produced using low-input living creatures, convey much basic supplies and yield to the environment (Hill et al. 2006). Studies suggested that at present the corn ethanol application is less petroleum-exhaustive, considering the cleanest liquid fuel apart from fossil fuels. Cellulosic ethanol is regarded as likely to substitute the first procreation biofuel made from agriculture crops, such as soybeans and corn. The effect of plant sizes surveyed that the approximate ethanol production

increase according to plant size for different biomass species. Lignocellulosic residues from agricultural wastes, forestry residue, and municipal wastes are explicitly plentiful in and around us and are capable of bioconversion (Sanchez 2009).

Yet, use of cellulosic alcohol is not practical on a commercial scale, but this practice could avoid usage of conventional biofuels, which are produced for food purposes. After much research, it was found that corn ethanol application was less petroleum-intensive and the huge land use for ethanol production for fuel purpose will subsequently require cellulosic application. Ethanol obtained from the cellulosic mass of living, is checked for a large-scale transportation fuel, but the environmental jolt cogitations are hindering the extensive utilization of fuel ethanol delivered from cellulosic biomass due to the change financial matters (Lynd et al. 1991). The promising alternative is the conversion of cellulosic mass. The technology to generate cellulosic mass from ethanol resources such as forestry and agriculture residue has not yet been viewed commercially. To generate cellulosic mass for bioethanol, treatment before use is to be followed, the cellulose part is hydrolyzed by enzymes or acids. Single origin of cellulosic biomass with the help of advance technology was protracted and enzymatic hydrolysis operation using identical analytic was done and it gave a comparative performance data developing sugar recapture from hemicelluloses and cellulose (Wyman et al. 2005).

Pretreatment is needed to develop the effective and mechanism models for cogent design and remove structural and compositional hindrance to hydrolysis in structure to make better frequency for enzyme hydrolysis and increase yield of fermentable sugar from cellulose or hemicelluloses (Mosier et al. 2005). Ionic liquids (ILs) did show an effective solvent for pretreatment of lignocelluloses, advancing the rapid hydrolysis of soluble polysaccharides to form simple sugar of cellulose and hemicellulose. The conversion of sustainable cellulosic biomass to low cost fermentable sugars in a biological, economic variety of valuable product should be made utilizing metabolic designing advancements and process development with industrial efforts and academic with fundamental knowledge and cost effective. Conversion economy is a crucial requirement to overcome research-driven improvements and cost competitive process for future prospects.

Observation founded that ethanol production occurs by the use of white rot fungus of cellulosic biomass as *Lenzites betulinus, Ceriporiopsis subvermispora, Dichomitus squalens, Plurotus ostreatus* and *Coriolus versicolor* naturally causes the need of titers of cellulases necessary for the simultaneously saccharification of pretreated lignocellulose or ethanolysis to show up the system of cellulosic part and reduce by breakdown of hemicelluloses to sugar. *Lenzites betulinus* fungi taken from the fruiting body of dead trees produce ethanol and exhibit various sugars content. *L. betulinus* is equipped in creating ethanol straight from rice straw and corn stalks. *Trametes versicolor* is found promising to directly produce lignocellulosic biomass and is environmental friendly to produce ethanol and demonstrated favorable potential to convert xylan into ethanol and non-pretreated starch and rice straw to recombinant strains. Yet cellulosic ethanol is one of the most desirable application options obtainable to lower the transmission sector of greenhouse gas emission. *Manila*, *Neurospora*, and *Fusarium* genera having filamentous fungi were

observed to show capacity of organisms to convert cellulose directly to ethanol. *Monilinia* a filamentous saprophytic fungus can be used by many polysaccharides and is capable of producing cellulose, fermented xylose, glucose, and direct ethanol from cellulosic material (Gong et al. 1981). In filamentous fungi, Lignocellulosic materials in huge amount where the cultivating residues are available which brings up problem of losing of capable and great deal of a specific kind of matter with the similar properties as biomass fuel production, paper manufacture, human and animal consumption among others and composting also in ejection of the environment. The capability of fungi to reduce the lignocellulosic substances is because of their enzymatic mechanism. Fungi such as *Trichoderma* sp. and *Aspergillus niger* release huge amounts of extracellular cellulolytic enzymes. *Trichoderma reesei* is found as an important source of commercial sources of cellulases and hemicellulase utilized as a part of depolymerize biomass to biofuels as ethanol. *T. rice* delivers and secretes plenteous amounts of enzymes that degrade the cellulose and related biomass parts. *Trichoderma harzianum* a filamentous fungus also makes cellulose degrading enzymes for achieving enormous profits of biomass usage and is used in industries feedstocks, with agriculture and forestry residue, woody biomass, dead trees, corn stalk of low value fibre is used in production of bioethanol (Agbor et al. 2011). Choices made for materials should meet the standard of sustainability with biofuels of current emerging engine designs. Table 13.5 illustrates the various microorganisms and substrates involved in the cellulosic ethanol generation.

13.4 Various Policies and Challenges

Various reasons are found for the use biofuels to be taken into account as closely related to technologies by more advanced and industrialized countries. The productivity of substitute biofuel policies in accomplishing vitality, ecological and agrarian strategy question are assessed utilizing financial money saving advantage examination (Gorter and Just 2010). Present energy policies addresses matter, including environmental friendly options to enlarge energy and contribute to give support for cleaner and more producing energy use (Demirbas 2009). With the current increase in oil prices and with increased concern about global warming caused by carbon dioxide emissions, biofuels had gained popularity (Ljungdahl (2008). Biofuels are

Table 13.5 Microorganisms and substrates involved in the cellulosic ethanol

Fungal strains	Substrates	References
T. reesei	Wheat straw	Chahal et al. (1985)
Piromyces sp.	Maize stem	Ljungdahl (2008)
Anaeromyces mucronatus 543	Orchard grass hay	Lee et al. (2001)
Neocallimastix frontalis	Cotton fiber Wheat straw	Doi (2008)
Orpinomyces sp.	Wheat straw	Chen et al. (1998)

environment friendly and the usage of biofuels would address global concerns of carbon emissions. The policy of biofuel is likely to increase its usage as transportation fuel produced from biomass and from other renewable resources (Demirbas 2009). Biofuel energy gives a security in environmental concerns, socioeconomic development as it is based on agricultural production and also saving of foreign exchange. However, cultivation of crops for production of fertilizers and pesticides and for manufacturing biofuel exerts a lot of energy. The economists have a strong effect on biofuels in the short-term is determined on variance components as harvest, oil price, increase in the economy, and level of complete list of items. Sustainable development is determined according to factors such as sound environmental practices, efforts in technological change, economic growth, efforts against climate change, and long-term policies toward energy, agriculture, and the environment (Rajagopal and Zilberman 2008). The study reported that the future energy can significantly increase with the use of second generation biofuels, however the economy is the major constraints to the commercial production (Carriquiry et al. 2011; Lamers et al. 2012) an important exporting countries have low feedstock costs and existing wood processing ventures and is greatly influence by policy structure (Lamers et al. 2012) to regenerate the economy by increasing demand and prices for agricultural products. Corn based ethanol does not meet the need of United States, though for commercial production for biofuel is advanced (Drabik and de Gorter 2011). Research is needed to provide further solution for the unofficial markets and for rapid commercialisation. Direct land use has a great impact on greenhouse gas emission and of eutrophication for all biofuels have been founded from the studies, the major importance in some cases is of the technical design of production. In the current situation the indirect land use change of biofuel were the situation of scientific uncertainty for the policy making, but the indistinguishable problem has been tackled in past (Di et al. 2012) the change of indirect land use effects the biofuels and reduces the benefits of that of fossil fuels.

The transformation of uncultivated land to biofuel farming brought about critical soil organic carbon loses (SOC) (Teixeira et al. 2009). Biofuel dispensation opened new career opportunities for people in rural areas. Biofuels are anticipated to lessen the reliance on imported petroleum. With land utilization and climatic change, cellulosic vitality could bring about new frontier into wide based field. The present and future can be alleviated through innovative technologies and suitable approaches that can fortify sustainable use of land by the indigenous locality for utilization of natural resources (Sawyer 2008).

13.5 Impact on Environment and Health

Energy we use today has been collected and stored by the process of photosynthesis (Bassam 2012) it has been encouraged for the research of renewable transportation biofuel related to petroleum contribution so that the negative environmental outcome of fossil fuels is reduced (Hill et al. 2006). Transmission of biofuels if created

from low-input biomass on agricultural area or from squander biomass, could give much extensively benefit to the environment food security and biofuel feedstock crops turns up for the rising competition with biofuel feedstock crops and food crops conversion process utilizes little fossil fuels which becomes more sustainable and not harming the environment (Granda et al. 2007). Land utilize changes in regards to deforestation forms, fertile land transformation and related methodological difficulties and new idea of land utilize markers has reverberated in biofuel creation is a modern phenomenon and it had not yet been incorporated as a figure driving forward change in land use. Changing in the harvest generation, particularly with the expansion request of feedstock crops for biofuels, has an alternate ramification of the expected patterns of land use under the mandates and trade liberalization scenarios substantial costs on society has environmental impacts of energy use (Hill et al. 2009). With the increasing of cost of food and human feed resources the production of ethanol fuel is the preference of other food crops as corn should be for food and feed. Creation of biomass for biofuel on the cost of circuitous land utilize might be the outcome as unmeasured natural effects of biofuels (Hill et al. 2009). The present cycle evaluations normally communicates that bioethanol gives out in decreasing of advantages being utilized and a worldwide temperature alteration, however the impact in fermentation, human and biological dangerous, creating fundamentally in developing and delivering biomass were for the most part of negative (Blottnitz and Curran 2007).

13.6 Current Status

Biofuels have been used for a significant timeframe as a way to deal with fabricate essentialness autonomy, diminish import costs, and its strengthen in changing (Araújo et al. 2017; Kovarik 2013). Since 2000, the general biofuels supply has extended up to 8% to meet the need of 4% of the world's vehicle demands in 2015. These gigantic rising is credited to plans, for example, blending directions, which develop more conspicuous utilize and may to some degree secure biofuels in the midst of time.

In enterprises, for example, flight, marine transport, and overwhelming cargo, biofuels are regarded to be the main functional, low-carbon contrasting option to petroleum product. Particularly aeronautics, greenhouse gas (GHG) outflows were anticipated to increment by 400–600% between 2010 and 2050, in view of anticipated development. In conjunction, the avionics business set its focus on diminishing CO_2 and flew their first business dry run with biofuels in 2008. As of mid-2015, roughly 22 aircrafts had finished in excess of 2000 traveler flights with biofuel speaking to up to a large portion of the stream fuel blend. Taking a gander at expansive numbers and sorts, the worldwide biofuel supplies rose to around 35 billion gallons in 2015 comprising generally of a 3:1 breakdown of ethanol to bio-diesel.

13.6.1 Interest in Biofuels

Looking past the specialized viewpoints, supportability/execution, and arrangement parts of biofuels, another basic measurement of the biofuel standpoint can be found in venture patterns. Worldwide interest in biofuels was assessed to measure up to $3.1 billion by 2015, mirroring a decrease of 35% with respect to 2014 and over 80% in ostensible terms since 2008. Towards the start of the twenty-first century, billions of dollars were invested by global oil companies to discover a cutting-edge biofuel, with considerable risks being undertaken in the process (Hochman 2014). Commercialization of cutting-edge biofuels is more expensive and elaborate than initially foreseen. The steep fall in oil price per barrel from $115 in June 2014 to $27 in 2015 recuperated to approximately $50 in the greater part of 2016, yet the unavailability of a lucid biofuel strategy in the United States until 2015, or more essential time and financially, have consolidated to dissuade everything except a little arrangement of speculators (Hochman 2014).

13.7 Conclusion and Future Prospects

Biofuels are the need of the hour in terms of environmental safety, depletion of fossil fuels, and increase in fuel prices. Microbe-mediated biofuel production is promising owing to its great metabolic efficiency and the plethora of biofuels they are capable of producing ranging from biodiesel, bioethanol, biogas, to syngas, to name a few. Fungi can be a good source of biofuels and are explored globally for generation of biofuels. Innovative strategies, employment of biotechnological tools, synthetic biology, metabolic engineering, and so on can lead to rapid commercialization of fungi-based biofuels for a sustainable future. Endophytic parasites verbalize with a promising wellspring of incipient age biofuels. Their lipid profiles and capacity to engender volatiles need to be explored extensively. The genome of the filamentous parasitic endophyte *Ascocoryne sarcoides* that engenders potential-biofuel metabolites when developed on cellulose medium has now been efficaciously described utilizing transcriptomic and metabolomics information (Gianoulis et al. 2012). Till date more sizably voluminous part of the endophytic fungi with biofuel engendering faculty have been found from one of a kind natural circumventions over the world, be that as it may, the Indian part of a great land mass is yet to be deliberately investigated for novel biofuel makers. The shifted geology and climatic conditions cumulated with gargantuan biodiversity accessible in India, makes it a potential fortune trove of novel biofuel distributing endophytic growths. Coordinated endeavors from the business side, colleges, and research institutions the nation over can enable India in playing a key role in the exploration of endophytes for cutting-edge biofuels.

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Chapter 14
Lignocellulosic Biomass to Fungal Oils: A Radical Bioconversion Toward Establishing a Prospective Resource

Batul Diwan and Pratima Gupta

14.1 Introduction

Lipids, fats, and oils have always been an inevitable social, commercial, and livelihood requirement. Lipids in general are also essential for growth and endurance of living organisms. Among their wide array of applications, food and fuel are undisputedly the fundamental ones and hence of highest priority. Upsurge in demand for renewable fuel (biodiesel, bio-oil, bio-crude) is subsequently increasing the need for lipid resources. However, demand for these lipid resources in the form of conventional oils such as vegetable oils and fish oils in food and nutrition industries is gradually outpacing the supply. Moreover, with the increasing awareness of health and nutrition, importance of functional and essential fatty acids is also on the rise. Fatty acids of different chain lengths decide the functionality of oils for serving various genres of applications such as feedstock to fuels or as a source of nutrition and nutraceutical. From a nutritional perspective, the dominantly recognized functional fatty acids are long-chain mono or poly-unsaturated fatty acids (PUFA such as ω-3, ω-6, ω-9 fatty acids) as well as medium-chain fatty acids (MCFA). However, regularly consumed vegetable oils typically lack in higher functional fatty acids. Therefore, finding an alternate source, which is rich in structural and functional fatty acids, is essential for serving both food and fuel purposes. In past two decades, research on microbial oils (MO) also referred to as single cell oil (SCO) as an alternate lipid source has accelerated, given its potential to serve both fuel (because of similarity to vegetable oils) model and nutritional (rare natural source of functional fatty acids such as PUFAs and MCFA) model applications. This chapter gives an introductory overview of SCO and its candidate microorganisms particularly fungal sources and their emergence as an important alternate oil source. It covers in detail

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the feasibility of lignocellulosic biomass (LCB) as potential substrates for SCO fermentation, real-world applications of SCO, factors influencing the LCB to SCO conversion and moves toward realization of the biorefinery concept.

14.2 Single Cell Oils

14.2.1 What Are Single Cell Oils?

The SCO refers to the microorganisms which have the potential to excessively accumulate intracellular lipids under certain sets of physiochemical and cultivational stress (Ratledge 1974). Earlier these nonconventional sources were considered solely for consumption purposes and hence included only oils having compositional similarity to vegetable oil, fit to be edible (Ratledge 2005). However, its boundaries have expanded in the past few years integrating different categories of lipids like algal, fungal, and even bacterial (Ratledge 2013) and are now among the well-known biotechnological products. SCOs are being recognized for their important contributions as a nonconventional source of rare long-chain PUFA. But now they are also drawing attention as a source of unusual functional fatty acids like MCFAs (Diwan and Gupta 2018a), which can play a key role in health and nutrition of infant as well as adults (Bach and Babayan 1982). Despite these exceptional properties, they have not acquired enough acceptance as a potential oil and fat source because of concerns of high cost involved for processing. In spite of growing global consumption trends of microbial products (yeasts and bacteria) in the form of flavored curds, beers, yoghurts, cheese, single cell protein (SCP), fermented delicacies, etc., the public acceptance of SCO as an edible product is still debated. Still, it is being believed that SCO would outdo its doubtable acceptability (at least as a candidate for health and nutrition) because of the potentials and possibilities it has to offer.

14.2.2 Oleaginous Microorganisms: The Candidates of SCO

14.2.2.1 Definition

The concept of oleaginicity originally came into light three to four decades ago. Scientists like Ratledge, Boulton, and the pioneers in the field validated the microorganisms, irrespective of their kingdom and genera, of having susceptibility to oversynthesize minimum 20–25% of their cell mass as intracellular storage lipids, as oleaginous (Boulton 1988; Ratledge 2013). Various genera and species of microalgae, bacteria, and fungi (yeasts and molds), which can accumulate lipids over 20% of their cell dry weight (CDW), fall under this category (Ratledge 1974). According to Subramaniam, four bacterial genera having lipid content from 24% to 78% CDW, four yeast and mold genera each with a reported lipid content ranging between 58–72% and 57–86% of CDW, and 14 genera of microalgae with lipid content

ranging between 20 and 77% were included under the category of oleaginous. The highest reported in each category were *Arthrobacter* sp. among bacterial genera, *Rhodotorula* and *Mortierella* among fungi (yeasts and molds), and *Schizochytrium* among microalgae (Jin et al. 2015).

In heterotrophic growth conditions, fungi, marine consortia, and microalgae generally produce more lipids to have commercial applicability. Normally the algal lipid content varies from 20% to 50%; however under certain set of conditions, it can reach up to 70–90% (Metting 1996). To name a few, *Chlorophyta and Bacillariophyceae* specifically *Chlorella*, which have shown high oil accumulation, have provoked huge interest in international bioenergy research and development. However, this chapter focuses on MOs from yeasts and molds; henceforth oleaginous fungi will be the topic of discussion. *Aspergillus* and *Mucor*, *Lipomyces starkeyi*, and *Rhodotorula glutinis* are some of the earliest discovered oil-accumulating fungi. Later yeasts like *Cryptococcus albidus, Lipomyces lipofer, Lipomyces starkeyi, Rhodosporidium toruloides, Rhodotorula glutinis, Trichosporon pullulans,* and *Trichosporon fermentans* and molds like *Aspergillus terreus*, *Claviceps purpurea*, *Tolyposporium*, *Cunninghamella elegans, Mortierella alpina, Mortierella isabellina, Mortierella vinacea, Rhizopus oryzae,* and *Thermomyces lanuginosus* were successively found to have oleaginicity (Liang and Jiang 2013).

For SCO, fungal source also has the advantage of higher growth rate and less freshwater expense over algal oil production. Molds have been mostly explored for special PUFA rich lipids like DHA (docosahexaenoic acid), GLA (gamma-linolenic acid), EPA (eicosapentaenoic acid), and ARA (arachidonic acid). Yeast has been more recognized for its higher production of bulk lipids. It is more popular for SCO production because of its unicellular characteristic, having high duplication rate, ease of lab, as well as commercial scale fermentation, and least susceptibility to produce endotoxins (Ageitos et al. 2011). Hence, they are considered as model organism for basic and applied researches in this field. The oils they produce are generally composed of triacylglycerols (TAGs) made of a variety of fatty acids (FA) similar to plant oils which opens the opportunity to use them as biofuel raw material.

14.2.2.2 Mechanism of Production

Apart from the physical aspect (20–25% oil accumulation), the biochemical aspect also originated with the belief that enzyme ATP: citrate lyase (ACL) is a marker of oleaginicity (Boulton and Ratledge 1981), and most, if not all, oleaginous fungi were found to possess this enzyme. The other important enzymes found to play a key role in oleaginicity are malic enzymes (ME) and acetyl-CoA carboxylase (ACC). In the event of any essential nutrient like nitrogen or phosphorus deficit, all these enzymes collectively come into play and trigger in shifting from usual metabolic pathways solely toward lipid synthesis ensuing into lipid accumulation. Fungal machinery in such occasions ceases to perform the regular intracellular functions like nucleic acid and protein synthesis, leading to arrest in cellular growth (Papanikolaou and Aggelis 2011). Availability of immediate pool of excessive

carbon is another mandate in order to maintain consistent lipid synthesis. With the beginning of nitrogen starvation, the microbial machinery starts activating the alternate supply of nitrogen by converting the available AMP (adenosine monophosphate) into IMP (inosine monophosphate) via enzyme AMP deaminase. The NH_4^+ liberated from this reaction compensates the growing intracellular nitrogen deficit. In due course, the intracellular AMP pool depletes which in turn inactivates another important AMP-dependent enzyme of TCA cycle enzyme, NAD+ isocitrate dehydrogenase (NID). Since NID is involved in conversion of isocitric to α-ketoglutaric acid, its inactivation results in accumulation of unconverted isocitric acid. Isocitric acid exists in equilibrium with citric acid hence an intra-mitochondrial citric acid reserve starts building. When the concentration reaches a saturation point, this citric acid is transported out from mitochondria to cytoplasm in exchange of malate via citrate-malate shunt. Enzyme ACL now marks the beginning of first committed step in lipid synthesis by cleaving this citrate into acetyl-CoA and oxaloacetate.

On the whole, the excess carbon substrate is initially being converted to pyruvate (through glycolysis and pentose phosphate pathway) which is converted into acetyl-CoA and citrate to participate in TCA. After a series of reactions, a pool of unconverted citrate grows in cytoplasm which is converted into acetyl-CoA. Hence broadly speaking, under essential nutrient limitation, the microbial machinery is channelizing available surplus carbon and transforming it into acetyl-CoA pool, the precursor of de novo lipid synthesis. The acetyl-CoA through fatty acid synthesis (FAS) pathways gets converted into fatty acids. This FA elongates to produce a variety of fatty acids of different chain lengths which eventually assemble to generate TAGs by commencing the process of lipid accumulation. In non-oleaginous fungi, this surplus carbon is definitely transformed into citrate through usual cascade of events; however, the stocked citrate in absence of ACL is either secreted out or makes its way to polysaccharide synthesis pathway. The stepwise progression from nitrogen starvation to lipid accumulation is shown in Fig. 14.1.

14.3 Advantages and Limitations of Conventional Oils

SCO has a long history of evolution from a being a subject of mere academic interest to becoming a theme of rigorous research as an essential source of unusual fatty acids (PUFAs, MCFAs) for health and nutraceuticals. Paucity of a safe natural resource, because of its absence in plant oils, led to this realization, and these mysterious microorganisms in due course gained attention. Meanwhile, expedition on converting SCOs to biofuels (biodiesel) also paced up. The collective reason for these transitions is because of the advantages associated with the microorganisms, such as fast growth rate, non-requirement of large tillable lands compared to oil crops and less seasonal and climatic dependence (Zhang and Hu 2014). The compositional similarity it shares with conventional vegetable oils (Wei et al. 2015) made it a suitable feedstock for fuels. Further, these microorganisms have the ability to consume a variety of substrates including synthetic, complex and organic like

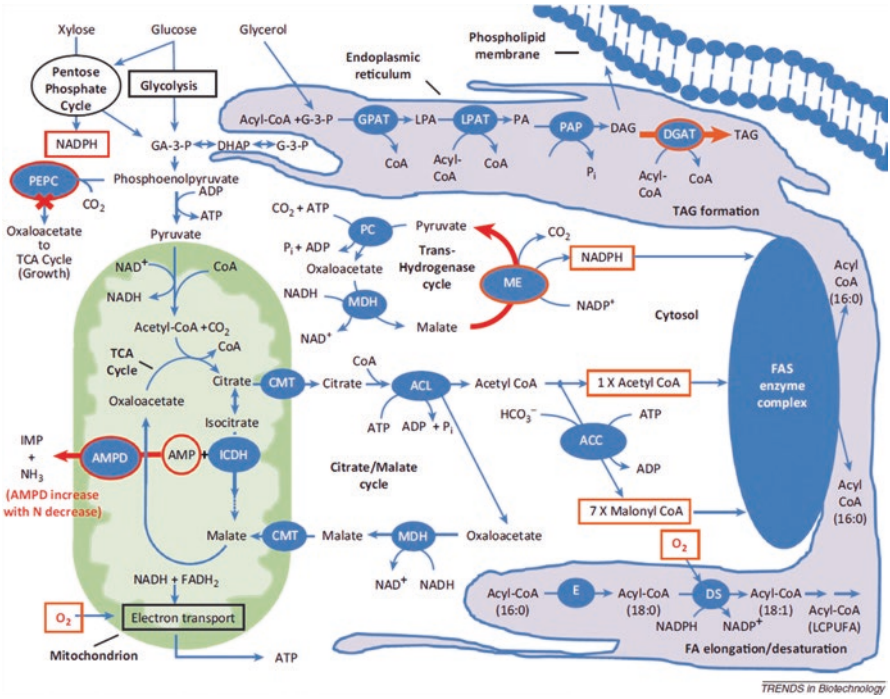


Fig. 14.1 Major cellular pathways, enzymes, control points, and organelles involved in the conversion of carbohydrates to lipids. Key acetyl- and malonyl-coenzyme A (CoA) substrates and NADPH cofactors supporting the conversion of carbohydrates to lipid are highlighted in red rectangles. Enzyme steps that have been consistently demonstrated to have a major impact on increasing triacylglyceride (TAG) biosynthesis are highlighted in red circles. Thick red arrows indicate upregulation of enzyme activity, while a red cross indicates downregulation observed to cause enhanced TAG formation. *ACC* acetyl-CoA carboxylase, *ACL* ATP:citrate lyase, *AMPD* AMP deaminase, *CMT* citrate-malate translocase, *DAG* diacylglyceride, *DGAT* diacylglycerol acyltransferase, *DHAP* dihydroxyacetone phosphate, *DS* desaturase, *E* elongase, *FA* fatty acid, *FAS* fatty acid synthetase, *GA-3-P* glyceraldehyde 3-phosphate, *G-3-P* glycerol 3-phosphate, *GPAT* glycerol 3-phosphate acyltransferase, *ICDH* isocitrate dehydrogenase, *IMP* inosine monophosphate, *LCPUFA* long-chain polyunsaturated fatty acid, *LPA* lysophosphatidate, *LPAT* lysophosphatidate transferase, *MDH* malate dehydrogenase, *ME* malic enzyme, *PA* phosphatidate, *PAP* phosphatidate phosphatase, *PC* pyruvate carboxylase, *TCA* tricarboxylic acid. (Taken with permission from Jin et al. (2015))

agro-industrial wastes to produce an array of metabolic products. Hence, these low-priced substrates can be employed to cut down the overall production costs.

The products can be tailored, modified, and improved qualitatively as well as quantitatively by necessary genetic modification of microorganisms. Moreover, in microbial fermentations, the process can be efficiently controlled for getting a desired output. The richness of SCO in functional fatty acids like PUFAs and MCFAs with relatively high oxidative stability than fish oils (the contemporary and richest source of ω fatty acids) can thereby make them a realistic nutraceutical sub-

stitute. Efforts are hence increasing not only on designing and strategizing the process but also on parameters affecting the process and the product yield. However, the high process economy associated with the large-scale production and high product yield required to suffice the need to convert into fuels are the major reasons for its slow acceptance apart from the issue of public acceptance. Such microorganisms will be grown heterotrophically using synthetic or organic carbon source which will be eventually consumed and converted to oils. But unlike plants, the SCO production dynamics depends on highly imbalanced culture conditions, where an essential nutrient like nitrogen or phosphorus exhaustion is mandatory. The starved nutrient should be concomitantly balanced with excess fermentable carbon in order to maintain both growth and lipid synthesis. Now maintaining such complex culture conditions in prolonged fermentative bioprocess like SCO itself gives rise to initial complexity. Further, the conversion efficiency of microorganisms is far imbalanced, almost 5:1 carbon to oils, which spontaneously increases the production cost (Ratledge 2005). Therefore, it is preferable to focus either on finding or producing either value added oils marginally exceeding the price of available edible oils or using low-cost carbon substrates for probable processing to reduce cost during large-scale production.

14.4 Wastes to SCO

Technological progress, global population explosion, urbanization, and industrialization have collectively intensified the scale of global waste generation both of degradable and nondegradable nature. According to prediction of World Bank, the waste generation is going to amplify further, and the figures would almost cross two-and-a-half billion tons by 2025. Management and safe disposal of wastes at one point was obligatory, but now it has become an economic accountability, more for developing than developed nations. World Bank report suggests that on an average US$20–250 per ton itself is expended in waste collection and US$20–350 per ton on disposal and dumping. Such waste generation, whether solid or liquid, is consistent throughout the year. Agricultural waste generation although might differ seasonally, but the overall contribution from the agricultural sector remains uniform year-round. Some of the regularly performed waste disposal practices like landfills and incineration generate byproducts and secondary waste like CO_2, methane, acid gases, dioxins, furans, as well as particulates, most of which can instigate the chain of other health and environmental hazards (Scarlat et al. 2015).

However, some of them like methane can be again valorized, and at many waste dumping sites, such realistic measures have been already taken which encourages capitalizing other wastes also. Hence, there is awareness nationally and internationally to shift toward biodegradable materials. Increasing anthem of reduce, reuse, and recycle has subconsciously provoked maximal utilization of available resources and value addition to generated wastes. Among a wide array of such wastes, agricultural wastes can be of special significance because of their organic makeup which

can serve as potential carbon source and their consistent availability throughout the year in one or the other forms. Different prospects are being explored to harness them as they can offset the production cost, effectuate safe waste management and alongside add value to it. Therefore, utilization of agricultural wastes for generating wide range of biotechnological products, be it in form of energy, fuel, or other valuable metabolites through microbial bioprocess, is on rise. Bio-hydrogen, ethanol, propanol, citric acid, phytase, surfactants, etc. (Cheng et al. 2008; Gupta and Parkhey 2015; Papanikolaou et al. 2008) are some well-known products which use agricultural wastes as substrates at lab as well as industrial scale. In the past few years, the exploitation of agricultural wastes for cost-effective SCO production is also being explored, but whether it can actually reduce the cost to a level where SCO can become an alternate to conventional oils is still questionable. Since other industrial and miscellaneous wastes don't seem to have much potential as substrate in terms of realistic conversion and product yield (Economou et al. 2015; Hwan Seo et al. 2013; Ren et al. 2015), the need is to exhaustively research every dimension in conversion of agricultural wastes to SCO. The challenges being confronted must be investigated and overcome so as to shape the SCO as a future alternate resource. The forthcoming section in this chapter is going to deal with such practical examples where agricultural wastes have been converted to SCO and the associated challenges and limitations to overcome.

14.5 Lignocellulosic Feedstocks to Bulk Lipids

Up until now the agricultural wastes were used for evaluating as substrates, among which the major ones being utilized in recent times are lignocellulosic wastes. The reason is its enormous worldwide production of around 150–170 × 10^9 tons (Pauly and Keegstra 2008) making it a ubiquitous resource and its carbohydrate backbone structure acting as a huge sustainable carbon reservoir. As the name suggests, it is composed of lignin, celluloses, and hemicelluloses. Celluloses and hemicelluloses are surrounded by the recalcitrant lignin which maintains its physical and compositional integrity and simultaneously results in a structural complexity. The cellulosic, hemicellulosic, and lignin contents of some of the common and abundant LCB are shown in Table 14.1.

Unless they are purposefully decomposed or degraded, they can be dried and stored for all-time availability. The lignin mesh prevents the chemical or enzymatic accessibility of core polymers for hydrolysis and therefore impedes their direct commercial viability. Hence, pretreatment of LCB is necessary to remove the lignin layers before the celluloses and hemicelluloses can be saccharified. Pretreatment also improves the porosity and decrease the native crystallanity of LCB. It can be carried out by any of the existing biological, chemical, electrical, physical, physicochemical, or combinatorial approaches (Kumar et al. 2009) depending upon the process cost and application. However with chemical pretreatment and sometimes saccharification, certain byproducts like furfural, hydroxymethylfurfural (HMF),

acetic acid, neutral and acidic phenolics, and various other chemicals are generated (Slininger et al. 2016). If not removed, they can inhibit microbial growth and production. These inhibitors can be neutralized and removed form saccharified LCB by implementing detoxification methods primarily through overliming (Huang et al. 2009; Yu et al. 2011). After carrying out all these range of treatments, any abundant LCB in immediate vicinity can be utilized suitably for other applications. Many LCB have made their way up to industrial scale as substrates for energy generation and fermentative metabolite production (Table 14.2). Now the feasibility of utilizing these feedstocks as potential substrate for lab scale as well as commercial SCO production has to be assessed.

Table 14.1 Composition of some common lignocellulosic feedstocks

LCB	Hemicellulose	Cellulose	Lignin	Other	Reference
Bamboo	24.6	46.7	28.1	0.6	Mui et al. (2008)
Corn Stover	26.3	43.3	13.6	16.7	Sun et al. (2011)
Rice straw	25	38	25	12	Diwan et al. (2018a); Taniguchi et al. (2005)
Soya stalks	17.3	37.6	25.4	19.7	Torgashov et al. (2010)
Sugarcane bagasse	27	45.5	21.1	6.8	de Moraes Rocha et al. (2011)
Sugarcane leaves	25	45	18	12	Singh and Chen (2008)
Switchgrass	26.1	33.48	17.35	–	Keshwani and Cheng (2009)
Wheat straw	28.24	43.68	8.25	19.83	Cone et al. (2012)

Table 14.2 Lignocellulosic biomass in industrial production commodity products

LCB	Biotechnological Firm	Country	Product
Molasses	Citrique Belge	Belgium	Citric acid, sodium citrate
Agricultural wastes	Ecomann biotechnology	China	Biodegradable thermoplastic, polyhydroxyalkanoate (PHA)
Straw, corn stover, wood, garden waste, and sugar cane bagasse	BioGasol	Denmark	High sugar hydrolysate for 2G bioethanol plants
lignocellulosic hydrolysates	Lesaffre advanced fermentations	France	Ethanol and other bio-based products
Corn cobs, corn stover, and bagasse	Praj	India	Ethanol
Agricultural wastes	Bio-on	Italian	Biodegradable thermoplastic, polyhydroxyalkanoate (PHA)
Corn or sugarcane residues	BioTork	US	Fuel ethanol, phytase, alcohol
Agricultural byproduct feedstock	Verdezyne	US	Dodecanedioic acid, sebacic acid, and adipic acid

14.5.1 Lignocellulosic Corn Byproducts as Substrates

Huge world annual production of around 1 billion tons of corn consequently generates millions of tons of byproducts like corn cob, stover, leaves, etc. These non-edible lignocellulosic residues can be realistic feedstocks that can be harnessed for cost-effective SCO fermentation (Table 14.3). Researches are being conducted on using corn byproducts as substrates for many well-known oleaginous fungal species. Upon using corncob hydrolysate as substrate yeasts like *Cryptococcus* sp. SM5S05 as well as *Trichosporon coremiiforme* responded well in lab scale investigations and produced around 7.6–7.7 g/l oils within 7–8 days of batch fermentation. The lipids were rich in both saturated and unsaturated fatty acids normally found in vegetable oils (Huang et al. 2013). Although substrate was the same, the corncob hydrolysate used in both studies has different concentrations of fermentable sugars; even then almost the same amount of lipids was produced. It is evident from this observation that the lipid accumulation is not just substrate dependent, but it also majorly depends on producer organisms and cultivation conditions.

Hemicelluloses, the fundamental constituent of every lignocellulosic biomass, are the next largest natural carbon reserve second to cellulose (Diwan et al. 2018b). Exploiting it can maximize the feedstock usage and its resourceful conversion, in a way that it will benefit the overall economy of SCO bioprocess. Hence, many studies investigated the simultaneous utilization of hexoses alongside pentoses (usually generated from hemicellulosic fraction) by oleaginous species. In one such case, both acid- and alkali-pretreated hydrolysates of corn stover containing both glucose and xylose in different ratios were used as substrate, and mold *M. isabellina* co-utilized both sugars resulting in 4.8 and 2.5 g/l lipids, respectively (Ruan et al. 2012). Similarly, yeast *C. curvatus* also displayed the ability of simultaneous consumption and conversion of hexose and pentose sugars from corn stover hydrolysate to produce 112 mg oil /g stover (Gong et al. 2013). In a very important study, ammonia fiber expansion (AFEX)-pretreated corn stover and acid-pretreated switch grass were used to screen lipid-producing strains. It was found that screened species produced substantially high lipid titer of around 25–30 g/l consuming all the available fermentable sugars (Slininger et al. 2016). Yeast *Trichosporon cutaneum* also reportedly produced significant lipid titers ranging from around 12.3 g/l up to the highest reported 22.1 g/l in corncob hydrolysate as C source (Huang et al. 2013). These strategies adapted by Huang or Slininger can be of commercial importance as the lipid titers greater than 20 g/l have been seen which are significant to compete with contemporary plant commodity oil yield. Because of enormous worldwide production, the availability of corn LCB is also not a concern; therefore, these studies encourage to evaluate the reproducibility of the applied strategies in a large scale setup.

Table 14.3 Assessment on global availability and suitability of major agro-industrial feedstocks as raw material for SCO production

Agro-industrial waste	World annual production	Largest producing zone	Maximum reported SCO production	Comments on suitability	Remarks
Rice straw	741 mT annual global production generates 2000 mT straw	~500 mT from-Asian countries of which ~150–250 mT – from east Asian countries (Nguyen et al. 2016) (Shafie et al. 2014)	*T. fermentans* 11.5 g lipid per L (Huang et al. 2009)	Suitable as potential substrate for bulk oil production	Southeast Asian countries could be most promising region for conversion of rice residues to microbial oils
Rice husk	164 mT (projected value)	~140 mT from Asian countries of which south and east Asia is the largest contributor (Shafie et al. 2014)	–	Suitable potential substrate for bulk oil production	
Wheat straw	729 mT annual global production generates around 1100–1300 mT straw (Koopmans and Koppejan 1997)	EU-highest producer China and India 2nd and 3rd highest in the league	*C. curvatus* 5.8 g lipid per L (Yu et al. 2011)	Suitable potential substrate for bulk oil production	Europe and Southeast Asian region could be most promising for conversion to microbial oils

(continued)

Table 14.3 (continued)

Agro-industrial waste	World annual production	Largest producing zone	Maximum reported SCO production	Comments on suitability	Remarks
Corn/ maize Cob	~1.04 bT annual global production generates Around 273 mT cob (projected as per residue to product ratio) (Koopmans and Koppejan 1997)	US-highest producer China and Brazil 2nd and 3rd highest in the league	*T. cutaneum* 12.3 g lipid per L (Gao et al. 2014)	Suitable potential substrate for bulk oil production	US and South American region most promising for conversion of corn/maize residues
Corn/ maize stalk	~2bT (projected as per residue to product ratio) (Koopmans and Koppejan 1997)		25–30 g lipid per L (Slininger et al. 2016)	Suitable potential substrate for bulk oil production	
Corn/ maize husk	200 mT (projected as per residue to product ratio) (Koopmans and Koppejan 1997)		–	Suitable potential substrate for bulk oil production	
Soybean hull	307 mT world annual production generates ~15 mT hull (projected) (Blasi et al. 2000)	With 108.0 mT US is the leader followed by Brazil of which ~5.5 mT hull ~270 mT straw (projected) (Koopmans and Koppejan 1997) (Blasi et al. 2000)	–	Suitable potential substrate for bulk oil production	US region most promising for conversion of hull and straw. SCR's suitability is limited to local regions near the production area
Soybean straw	~770 mT (projected as per residue to product ratio) (Koopmans and Koppejan 1997)			Suitable potential substrate for bulk oil production	

(continued)

Table 14.3 (continued)

Agro-industrial waste	World annual production	Largest producing zone	Maximum reported SCO production	Comments on suitability	Remarks
Sugarcane bagasse	1.88 bT world annual production generates ~313 mT bagasse (projected) (Koopmans and Koppejan 1997)	With 736 mT Brazil leads in sugarcane production generating ~213 mT bagasse and ~33 mT molasses (projected values) Followed by India, China, and Thailand (Koopmans and Koppejan 1997) (Qazi 2014)	*T. fermentans* 15.8 g lipid per L (Huang et al. 2012)	Highly favorable substrate for bulk oil production	Most suitable raw material for SCO production in south American and Southeast Asian region of the world, while molasses can strictly serve as a substrate in region local to sugar distilleries
Sugarcane molasses	48.6 mT(projected) (Qazi 2014)		–	Favorable substrate for bulk oil production but suffers transportation and storage limitation	
Sugar beet pulp	270 mT world annual production generates ~148 mT Sugar beet pulp (projected) (Berłowska et al. 2016)	EU (France and Germany) is highest producer of beet pulp with 13 mT beet pulp generation and ~32 mT molasses (projected value) (Berłowska et al. 2016)	–	Favorable substrate for bulk oil production but suffers transportation and storage limitation	Suitability is limited to local producer regions
Sugar beet molasses	~11.3 mT (projected value) (Berłowska et al. 2016)			Favorable substrate for bulk oil production but suffers transportation and storage limitation	

Adapted with permission from Diwan et al. (2018b)

14.5.2 Lignocellulosic Rice and Wheat and Other Residues as Substrates

The global annual production of rice and wheat is more than 700 million tons (Table 14.3). When the grains are processed and marketed, it leaves behind millions of tons of non-edible husks and straw residues of lignocellulosic nature. Despite having unlimited exploitable worth, most of their portions are burnt in the fields

procedurally. However, minor portions are taken to the industries and are burnt to generate energy. The concerns of rising environmental pollution, especially air pollution, have alarmed most of the developing and developed nations to restrict the practice of burning unregulated agricultural biomass. India is one of the highest rice- and wheat-producing nations across the world; consequently the spare husks and straw being produced are also in abundance. Burning of these residues is creating serious environmental concerns especially in northern parts of the country. Hence, an National Green Tribunal Act was passed and a statutory body called National Green Tribunal was established 2010 years back to control such unrestricted burning. Since rice and wheat are staple crop in most of the regions of the world also, their residues are ubiquitously available everywhere. An excellent opportunity emerges here to transform them into value added products like microbial oils, which is also an environmentally safe method. Some practically useful evidences of wheat and rice LCB utilization as substrates can be found in literature which supports the possibility of its industrial scalability. Wheat straw and bran has been not only used in a liquid saccharified form but also in solid form for solid state fermentation (SSF). But it was found that even though highly active cellulases were employed for synergistic action, the approach did not result in significant yield and only about 10–18% of CDW was accumulated as lipids (Hui et al. 2010; Peng and Chen 2008). On contrary, when saccharified wheat straw was used after detoxification for submerged fermentation, several oleaginous fungi like *M. isabellina, M. vinacea, Cunninghamella elegans, R. oryzae, A. terreus, T. lanuginosus* produced significantly improved amounts of lipids (Zheng et al. 2012). Similarly in a study on five yeasts simultaneously, except yeast *R. toruloides* which was not able to tolerate non-detoxified wheat straw hydrolysate, the rest of the oleaginous species *L. starkeyi*, *Yarrowia lipolytica, Cryptococcus curvatus*, and *Rhodotorula glutinis* accumulated lipids using both detoxified and non-detoxified hydrolysate almost equivalently. Highest production achieved by *C. curvatus* was 4.2 g/l in detoxified and 5.8 g/l in non-detoxified hydrolysates (Yu et al. 2011). The reports make it apparent that using saccharified wheat straw or bran for submerged fermentation, i.e., liquid form of LCB, was much more effective than directly using the biomass for solid state fermentation. Yet the figures achieved were not significant enough compared to the highest oil production seen with corn LCB hydrolysates. In case of rice residues, yeast *Trichosporon fermentans* reportedly converted sugars from acid-saccharified followed by detoxified rice straw hydrolysate to produce 11.5–12.1 g/l in two different studies (Huang et al. 2014; Huang et al. 2009). A recent report also suggests the requirement of detoxification for oleaginous yeast *Trichosporon cutaneum*, which assimilated 38.2% cellular lipids corresponding to 6.7 g/l total lipids in detoxified acid barley hull hydrolysate and enzymatic hydrolysate (Guerfali et al. 2018).

On the contrary, another recent study showed high lipid content of around 40% CDW in non-detoxified compared to 26% CDW in detoxified rice straw hydrolysate by *M. alpina* (Diwan et al. 2018a). Similarly, 3.6 g/l lipids were produced by *M. isabellina* again in non-detoxified rice hull acid hydrolysate (Economou et al. 2011). It can be seen that acid-saccharified LCB usually requires detoxification before

being applicable as a carbon source for some oleaginous species. However, the hydrolysates are endured and utilized for lipid accumulation without any detoxification by some other yeasts and molds. Contrasting evidences suggest the different inherent tolerance capacities of different fungi for such complex substrates. Of the main inhibitory metabolites generated as byproduct of chemical pretreatments and saccharification like furfurals, HMF, acetic acid, phenolics, anomalously some like acetic acid, vanillin and HMF have been reported to act as oleaginous stimulators (Huang et al. 2011). This can also explain the abnormal behavior why non-detoxified hydrolysates were not only being endured but rather higher lipid accumulation was seen in some of the oleaginous species (Diwan et al. 2018a). The investigations and outcomes in a way supports that an entire step of detoxification can be eliminated sometimes, which can thereby reduce the overall cost of fermentable substrate preparation from LCB biomass. However, different LCB biomass can result in different degrees of fermentative inhibitors intrinsically depending on their respective structural composition.

Moreover, the tolerance and proliferative abilities in such medium also vary from organism to organism; therefore, strategies have to be carefully designed depending upon the process, organism, and LCB biomass chosen. Another noteworthy observation made from the reports is that the lipid titer is visibly more pronounced in rice residual LCB than in wheat residues. But huge availability of both of these residues presents a bright opportunity to utilize them on a commercial scale for producing microbial oils, be it neutral bulk lipids intended for production of biofuels or production of high value oils rich in unusual functional fatty acids. Being a byproduct of edible crops provides them additional advantage of being a safe substrate for production of such edible oils. The only aspect that needs to be addressed is average lipid titer, which is way less and insufficient when the question is about commercial viability of oil production.

14.5.3 Lignocellulosic Sugarcane Residues as Substrates

Sugarcane is among the leading agricultural commodities being produced worldwide. With 1.88 billion tons of yield, it is in the forefront of global annual production simultaneously producing generous amounts of bagasse to be valorized at liberty. Usually 40–50% of the generated biomass is being consumed for energy generation in distillery plants (Rabelo et al. 2011). The remaining unutilized bagasses would still be sufficient to exploit and converted into valuable chemical products through fermentative bioprocesses. They are abundant and economical and have ease of storability and transportability which adds to their promise as substrates for SCO production. Not only their bagasses but their industrial byproduct from sugar mills, molasses, can prove an exceptionally potential substrate. However, the chapter strictly focuses on lignocellulosic biomass as substrates; hence, possibilities and practicalities of molasses are not discussed here. In a related study with saccharified and detoxified sugarcane bagasse used as a C source, oleaginous yeast

Y. lipolytica produced around 11.4 g/l lipids within 96 h of fermentation. The hydrolysate was composed of both hexosic and pentosic sugars such as glucose, arabinose, and xylose, and the yeast co-utilized sugars for intracellular lipid accumulation resulting in a decent overall oil recovery (Tsigie et al. 2011).

In another report utilizing sugarcane bagasse hydrolysate as a substrate in a large scale setup for lipid production, oleaginous yeast *R. toruloides* produced lipids which were efficiently converted to biodiesel which obtainment up to 67% yield (Zhao et al. 2012). In another study purely on lipid production by utilizing sugarcane bagasse hydrolysate, *T. fermentans* produced remarkable 15.8 g/l lipid (Huang et al. 2012). Even though titers are less than the highest obtained with corn residues as substrate, they exceed any of the reports on rice residues, projecting sugarcane LCB residues as a promising feedstock for SCO bioprocess.

14.5.4 Miscellaneous Agricultural Residues as Substrates

In an agriculture-driven world, where major food and social requirements are fulfilled by agricultural commodities, generation of related municipal solid byproduct such as fruit peels, seeds, vegetable peels, leaves, and stalks occurs side by side. Therefore, instead of leaving them to rot in mere waste, these ubiquitous agro-residues can be strategically valorized to value-added products like SCO. In such practical efforts, sweet gum and detoxified pine autohydrolysate were used as carbon source for two *Rhodococcus opacus*, strains PD630 and DSM 1069, respectively, to result in lipid recovery of 0.25 and 0.31 g/l (Wei et al. 2015). Yeasts, *C. curvatus* and *T. cutaneum*, displayed lipid accumulation of 46% and 48% of CDW when they were grown on pectin, and beet pulp hydrolysates constituted of sugars like galacturonate and arabinose, respectively (Wang et al. 2015). Although experiments on such agro-residues resulted in poor lipid recovery, the possibility of using such substrates encourages further investigation and schematic process designed, for improving yield and economics.

From the discussion so far, it is apparent that LC residues such as rice straw, hull, wheat straw, husks, corn cob, stover, and sugar cane bagasse are far more abundant and substantially available compared to other miscellaneous agro-residues to take practical advantage. Further, the practical examples of flask scale as well as scaled-up LCB to lipid conversion have shown promising yields particularly with corncob hydrolysates. They have inherent benefits of stability and hence storability. Their recalcitrant physical structure, unlike highly degradation-prone agro-residues, facilitates their long-term accessibility and use. Since these are among the major staple crops, they are available in almost every major regions of the world like China, Brazil, Europe, India, Indonesia, Malaysia, Mexico, the USA, etc. facilitating their easy transport to any surrounding industrial region (Table 14.3). Moreover, all of them are byproducts of edible crops, offering the opportunity of using them for conversion to safe edible oils. All these facts collectively suggest them suitable to serve as suitable substrates for biomass to bulk as well as valuable lipids. The

challenges still underlying are low lipid content, yield and titer and endurance to non-detoxified hydrolysates. Although the yield obtained from corn residual LCB is commercially competitive, the yield using rice, wheat, and to some extent sugarcane residues requires significant improvement.

14.6 Lignocellulosic Feedstocks to Valuable Lipids

Valuable oils refer to the oils composed of not just usual C12 or C14-C18 or up to C20 fatty acids but other unusual functionally and nutritionally essential FA especially long-chain PUFAs which are extremely important for proper functioning of human body as well as for lifestyle disease prevention. Omega-3, omega-6, omega-9, etc. are some of the well-known groups of PUFAs, classified on the basis of the double bond position from methyl terminus. Some of them, especially the precursor fatty acids for conversion into other long-chain PUFAs cannot be synthesized in human body which makes them far more essential. Alpha-linolenic acid (ALA) an ω-3 FA, arachidonic acid (AA) an ω-6 FA, dihomo-gamma-linolenic acid (DGLA) an ω-6 FA, DHA an ω-3 FA, EPA an ω-3 FA, GLA an ω-6 FA, and linoleic acid (LA) an ω-6 FA are few examples of ω-3 and ω-6 fatty acids. While most of the mammalian biofunctions are regulated by AA, ALA, and LA (Vadivelan and Venkateswaran 2014), ω-3 FAs like ALA, EPA, and DHA have shown positive effects in several physiological, immunological, and genetic disorders like asthma, breast and lung cancer, Crohn's disease, hypertension, and rheumatoid arthritis (Merendino et al. 2013; Vadivelan and Venkateswaran 2014). Studies have shown that average regular ω-6 FA consumption is much higher than ω-3 groups of FA to a level where ω-3 is almost absent from the diets (Sijtsma and De Swaaf 2004), and the trends are pretty much similar all over the world. This results in misbalanced ω-3: ω-6 intake ratio which in turn leads to enhanced inflammatory responses inside the body. The traditional sources of FA being most widely consumed are plant oils, among which canola, flax, hemp, soybean, and walnut are mostly supplying ALA. ALA an ω-6 FA is the precursor fatty acid to higher long-chain PUFAs and is converted in human body to EPA and DHA slowly (Gerster 1998). Also the conversion rate is sometimes not efficient to meet the daily requirement. Hence, to redress the generally believed optimal balance of both FA groups, the overall ω-6 intake must be reduced and replaced with equivalent introduction of ω-3 through an external alternative source. The most popular current alternate of ω-3 group PUFA recognized all over the world are fish oils. Being a highly acclaimed source, it is not only on the edge of demand to supply disparity but also is accompanied by rising price. Fish and fish oils are also sometimes contaminated with other antagonistic fatty acids and toxins like mercury. Moreover, fish production is reliant on seasonal and geographic conditions (Certik and Shimizu 1999). However, the sudden increase in awareness toward health and nutrition has also inflated the global fish consumption which is posing a greater ecological threat for the future. Therefore, it is important to explore and study other alternate, unconventional source of such FA which is not

associated with risk of originating any ecological imbalance. Algal and microbial oils including oils from marine phytoplanktons, lower fungi and few bacteria are the upcoming alternate for PUFA-rich oil sources (Ratledge 2001).

Literature also supports that a significant PUFA titer can be obtained from some well-recognized marine protists and algae. However, their cultivation at large scale is inconvenient and demands heavy water requirement, while marine microbial adaptation poses another difficulty in practically capitalizing them. These drawbacks encourage exploring other PUFA source like fungal oils. Molds belonging to various genera like *Cunninghamella, Mortierella, Mucor, Schizochytrium,* and *Thamnidium* (Bowles et al. 1999; Fan et al. 2001; Peng et al. 2011) and yeasts like *Yarrowia* and *Rhodosporidium* (Bellou et al. 2016) are some of the reported fungal species recognized for producing high PUFA titer. However, these studies were based on oil production utilizing expensive synthetic carbon sources which is the main reasons behind the probable high cost. Although these valuable oils would anyway be expensive than normal oils, replacing the synthetic substrates with inexpensive lignocellulosic biomass can offset the prize to some extent. The purpose of these oils is to serve in nutraceutical domain basically for improving the global health scenario, so the correct substrates selection becomes primary here and the cost secondary. Therefore, lignocellulosic biomasses safe for consumption become the next best alternative not only by being edible but by being inexpensive. In a study, *Cunninghamella echinulata* was shown to utilize different agro-wastes like corn gluten, corn steep, and tomato waste hydrolysate and produced a significant 800 mg/l GLA on tomato waste hydrolysate (Fakas et al. 2008). A promising fungus *Thamnidium elegans* was grown in a mixture of glucose and xylose mimicking a lignocellulosic hydrolysate-based fermentation medium. Co-utilization was observed, and the fungus produced lipids having high GLA content of around 1014 mg/l. The study supports the potential LCB valorization for production of oils with high nutritional and pharmaceutical interest (Zikou et al. 2013).

Use of lignocellulosic hemp hydrolysate was studied by a group of researchers on oleaginous marine protist *Schizochytrium*, and it was found that 59% of the total fatty acids produced was PUFAs mainly DHA (Gupta et al. 2015). Saccharified biomass of a cheap, non-food crop Jerusalem artichoke was utilized as a substrate for heterotrophic thraustochytrid *Aurantiochytrium* sp. which reportedly produced a significant 47% of its total lipids as DHA (Yu et al. 2016). Other than lignocellulosic biomass, some other miscellaneous agro-wastes are also generated in huge amounts as discussed in section "Miscellaneous agricultural residues as substrates" which can be harnessed efficiently in PUFA-rich oil production. Reports have shown utilization of these byproducts and wastes like barley, peanut bean residue, millet, wheat and rice bran, sweet potato, soybean hull, sunflower, and soybean pressed cake (Fakas et al. 2009; Ghobadi et al. 2011; Jacobs et al. 2009; Zhang and Hu 2012) by species of genera *Mortierella*, already recognized for production of various groups of PUFAs like AA, ALA, DGLA, EPA, and GLA. In another study, orange peel was used as a C substrate for *C. echinulata* which produced around 9% of its total lipid as GLA (Gema et al. 2002).

The apparent problem associated with these byproducts is their inconsistent availability labile to seasonal variations. Also they are prone to decomposition hence storability is an issue. Further, the PUFA titers reported in most of the study on these substrates are practically incompetent from the industrial point of view. However, an essential advantage they still have is that they can be safe from consumption aspect. Hence, the available amounts can be sufficient to capitalize for low volume production of these high value oils, which are any way required in miniscule dosage for consumption. The other way they can be taken advantage is by using them directly for solid state fermentation (SSF) completely bypassing the excessive freshwater investment. Also some of such edible substrates can be directly enriched in PUFA-rich lipids through this strategy (Čertík et al. 2013; Economou et al. 2010; Fakas et al. 2009; Gema et al. 2002) which later after necessary processing can be utilized either for consumption or as feed supplements. PUFA-rich oils can also be blended with other oils or added as emulsions to consumable items. Other feasible option is to use these SCO directly as food supplements similar to the SCP concept. Another group of functionally important fatty acids are medium-chain fatty acids ranging from C6 to C12 carbon chain. They are known to play vital role in dietetics, nutrition and medical sector. The main reason behind their functionality is their small size, greater solubility and rapid metabolism than long-chain fatty acids (LCFA), which endows them the property of easy digestibility in contrast to LCFA. Also in course of metabolism, LCFA are deposited as fats, while MCFA are readily oxidized to provide quick energy and are therefore great energy source without the risk of inducing obesity (Lee et al. 2012). Because of these important characteristics, they play an important role in obesity control and for other medical applications like carnitine system deficiency, epilepsy, hyperalimentation, serum cholesterol reduction, infant formula, and low-fat food alternative (Babayan 1981; Bach and Babayan 1982; Carnielli et al. 1994).

Structuring of tailor-made lipids with MCFA like caprylic acid and other functional long-chain FAs to design nutritionally important medium- and long-chain fatty acid (MCLT) with antiobesity properties has become the subject of interest. Most of the natural oil sources including conventional vegetable oils, cooking oils, and animal fats are only rich in long-chain fatty acids (C14-C20) and lack such kind of MCFA except for coconut, corn, and palm kernel oil. But a very recent research published in 2018 has shown that 80–90% of total lipids produced by two yeasts *Candida tropicalis* and *Pichia kudriavzevii* were MCFA-caprylic acid (Diwan and Gupta 2018a). The study suggests the possibility of SCO as a source of MCFA also. Researches are also ongoing to produce such MCFA-rich microbial lipids utilizing agro-wastes as inexpensive substrates, and some researchers have already shown progress in converting LCB to MCFA-rich lipids through the aforementioned yeasts (Diwan and Gupta 2018b). To aim for potential scale-up, lipid yield and titers of valuable FA like PUFA and MCFA need a great deal of improvement. Moreover, the abundance of suitable substrate in question should also be assessed before these oils can realistically become an alternate to algal or fish oils. Figure 14.2 pictorially demonstrates the concept of conversion of LCB and other industrial wastes to broadly applicable forms of SCO (nutraceuticals and biodiesel).

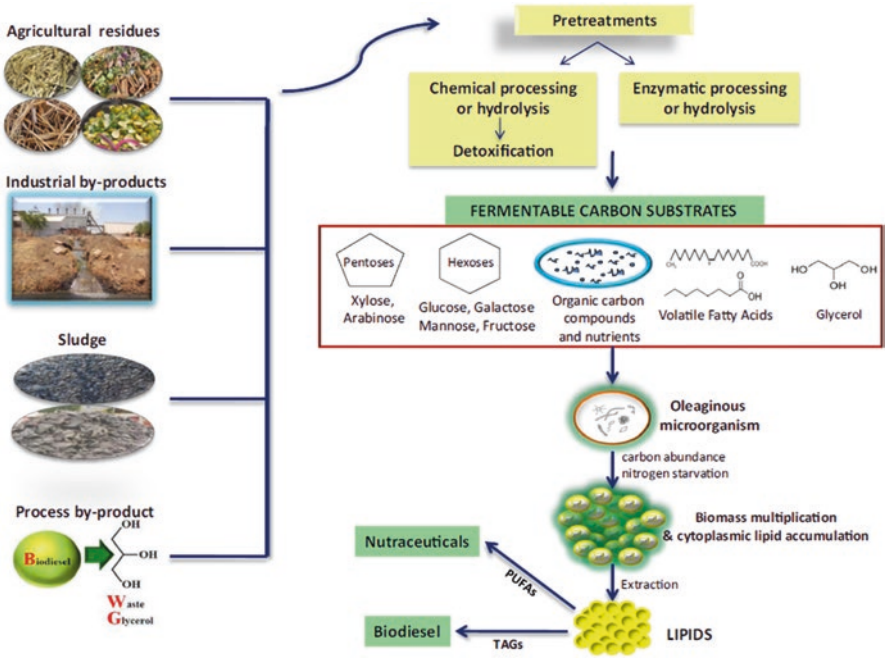


Fig. 14.2 A schematic representation of various steps involved in bioconversion of agro-industrial wastes to SCO. (Source: Diwan et al. (2018b))

14.7 Real-World Applications of SCO

Like plant oils, microbial oils are also mainly composed of TAGs and some free fatty acids. The majority of current research in microbial oils is focusing on utilizing them as raw material to renewable fuels because of two reasons: their compositional similarity to vegetable oils and their high ratio of carbon to heteroatom (Jin et al. 2015). Earlier it was thought that these lipids could only be converted into renewable fuel, i.e., biodiesel. But nowadays, other kinds of bio-based fuel and fuel alternatives have evolved such as renewable diesel and bio-crude. Biodiesel is a transesterified product of oils and fats, while renewable diesel is a hydrodeoxygenated version of oils and fats carried out at high temperature and pressure with the help of a catalyst (Knothe 2010). Bio-crude, sometimes also referred as bio-oils, on the other hand, is a kind of liquid fuel developed by thermochemical liquefaction such as hydrothermal liquefaction (HTL) of biomass in the presence of solvents under high temperature and pressure, with or without catalyst (Cheng et al. 2017). While composition of renewable diesel is supposed to simulate the petroleum-derived diesel fuel, bio-crude is under investigation as a substitute to petroleum fuel. Microbial oils can be fitting feedstock to these biofuels, while the oleaginous biomass can play an apt substrate for bio-crude generation. These high temperature and

pressure-mediated hydrolysis processes can transform the TAGs into microbial lipids to glycerol and FA with numerous further applications as shown in Fig. 14.3.

Produced glycerol byproducts are utilized for production of commercially important C3 chemical shown in Fig. 2b having widespread applications. Moreover, several functional fatty acids like EPA, DHA, GLA, and MCTs in microbial lipids discussed in previous section open other dimension of their applicability. Some of the oleaginous fungi have already been recognized and employed for commercial

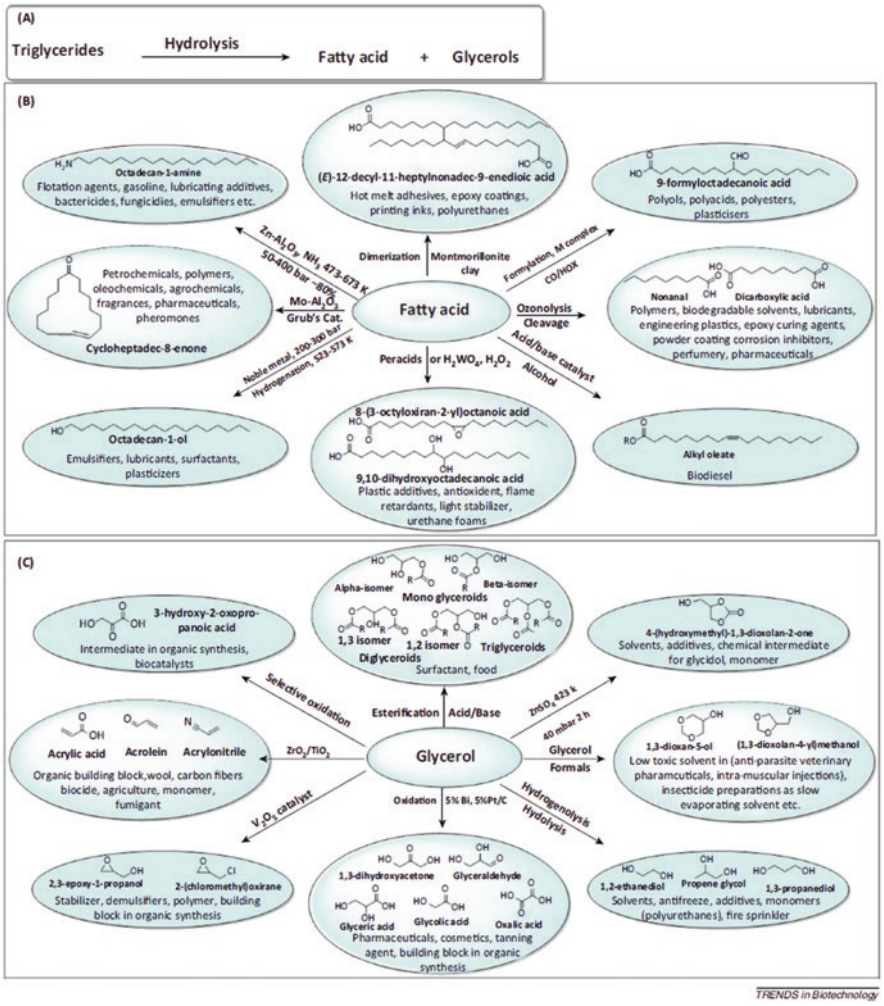


Fig. 14.3 Possible applications and transformations of glycerol and fatty acids derived from microbial lipids; (**a**) Reaction showing hydrolysis of TAG into Fatty acid and glycerol; (**b**) Multiple derivatives and applications of fatty acids released from TAGs present in oil; (**c**) Multiple derivatives and applications of glycerol released from TAGs present in oil [Taken with permission from Jin et al. (2015)]

scale production of PUFA-rich oils. Mold *M. alpina* is already in ARA production by Cabio Bioengineering Co.; J. and E. Sturge is producing GLA oils at its fermentation facilities by *Mucor circinelloides,* while E.I. Du Pont is manufacturing EPA-rich SCO by *Y. lipolytica*. Since SCO production is an expensive venture, practice of using cheap feedstocks for leveraging the process cost has been adapted even at commercial establishments. ChainCraft, a Netherland-based firm, is producing valuable medium-chain fatty acids using agro-wastes, with numerous biotechnological applications. AlgaVia is although working on commercial algal lipids, the approach of production is similar to heterotrophic fermentation like the case of fungal SCO. An upcoming biotechnological organization from India is commercially producing bio-crudes from algal biomass by hydrothermal liquefaction, and research is already under progress for bio−/petro-crude production by oleaginous fungal biomass. Since the overall low productivity of total lipids render their conversion into biodiesel practically an infeasible option, these microbial bio-crudes can offer a forthcoming renewable fuel substitute which can also be blended with algal petro-crude for realistic usability.

14.8 Factors Influencing Conversion of LCB to SCO

Although there is practical feasibility of establishing SCO as an essential oil and fat substitute, there exist numerous underlying challenging factors like high production cost primarily because of imbalanced culture conditions which demands the presence of excessively high fermentable carbons. To overcome this hurdle, utilization of LCB as inexpensive carbon source has lately gained huge research attention. But the conversion of LCB biomass to SCO is associated with several technical bottlenecks like poor intracellular lipid content and yield in conjunction with substandard productivity and titer. Each step of this conversion is associated with technical obstacles originating right from balancing high carbon to nitrogen during fermentation, selection of feedstock for balancing production and economy, maintaining cultivation conditions given the presence of mixed sugars and inhibitors in LCB hydrolysates, and substrate utilization and coproduction. Each of these factors has been discussed in detail to provide an integrative outline of the challenges and their possible realistic remediation measures.

14.8.1 General Logistics of LCB Feedstock Selection

There are several parameters governing the choice of substrate, namely, local availability, cost, long-term storability, and transportability. Regarding the storability, some of the carbon-rich feedstocks with high moisture or water content like beet molasses and corn molasses, cane, spoiled date syrups, sugar sorghum juice, and whey are much prone to degradation or autolysis. Although their storage can be

achieved using the recent advanced facilities, it will end up again in inflating the cost. Additionally, the transportation facilities for high moisture-containing substrates are also going to elevate the overall process cost and have other economic and environmental implications. Hence, if these are the substrates of choice in any SCO fermentation scheme, it would be preferable to utilize them in their immediate locality. On the contrary, these are the major advantages in employing solid lignocellulosic agro-wastes, as they are dry and structurally recalcitrant and have inherent advantage of storability, transportability, and hence all-time availability. However, the logistics operation in this low-density biomass originating from multiple harvesting, preprocessing, storage and transport, environmental effects of biomass, and generated product cost requires serious assessment in feasibility study of biomass to SCO conversion (Shafie et al. 2014). Local feedstock, which is year-round available, is an appropriate choice to maximally control the economy of the fermentation-based bioprocesses. The conversion of fermentable carbon to oils is not highly efficient in microbial system and statistics suggest this ratio to be around 5:1. Going through these stats, for meeting the daily minimum commodity oil requirement, i.e., 30 tons, 10,000 ton oil/year must be produced in turn requiring 50,000 tons of fermentable carbon. Hence the abundant and ubiquitous LCB discussed in this chapter like corn byproducts, sugarcane bagasses and leaves, paddy, and wheat husks and straws (Table 14.3), which have already gained industrial acceptance for production of other valuable commodity chemicals (Table 14.2), can be promising for SCO fermentation.

14.8.2 Fermentability of Complex LCB

Sometimes despite being inexpensive and locally abundant, a feedstock cannot be exercised as a substrate because the microorganism cannot suitably utilize them. The perfect example of this is cheap carbon-rich alkanes which became an ideal commodity chemical for industries in the past few decades. However, when employed as a cheap substrate for *A. niger*, the mold failed to utilize them for citric acid production. Hence, regardless of the low cost, it didn't prove to be an utilizable substrate. Similarly, the structural recalcitrance of lignocellulosic biomass makes pretreatment obligatory in order to make it a fermentable substrate for microorganisms. However, many a times these chemical pretreatment and saccharification strategies lead to generation of toxic microbial inhibitors which can also aggravate the microbial fermentability. Detoxification strategies have been invented to eliminate these inhibitors from saccharified biomass, but they alleviate the process complexity as well as economy. Use of robust oleaginous strain which can endure non-detoxified hydrolysates can relieve this situation by reducing the necessity of steps like detoxification for preparing a fermentable substrate. Some examples of such strains have been discussed in the previous section. The lignocellulosic biomasses are composed of two macro-polysaccharide chains – cellulose and hemicelluloses – saccharification of which generates abundant hexose and pentose sugars.

It has been seen that often the saccharification of LCB release relatively equal if not high titer of pentoses compared to hexoses (Yu et al. 2011). As per the general figures for metabolic conversion, each gram of xylose can lead to production of 0.34 g acetyl-CoA, while glucose produces 0.32 g acetyl-CoA (Papanikolaou and Aggelis 2011).

Reasonably, xylose (if metabolism follows phosphoketolase pathway) can lead to higher lipid synthesis than glucose if all the generated acetyl-CoA enters the lipid synthesis pathway. Therefore, co-utilization of pentose and hexose sugars during fermentation is essential, which can increase the efficiency of lipid conversion. This factor must also be considered while optimizing the bioprocess and microbial species having potential to utilize a variety of sugars, must be preferred. *Pichia stipitis* and *P. kudriavzevii* are some of the naturally occurring xylose fermenting yeasts (Agbogbo and Coward-Kelly 2008), while strain modification have also been carried out on species like *S. cerevisiae* for inducing pentose utilization (Bettiga et al. 2009; Li et al. 2016). Apart from glucose and xylose, other monosaccharides and disaccharides which can support growth and lipid synthesis are arabinose, galactose, fructose, mannose, sucrose, etc. of which most are available in one or the other LCB hydrolysates. However, the mixture of sugars can sometimes lead to the issue of diauxic growth effects. During saccharification, a few oligosaccharides are also generated along with monosaccharides. Some oleaginous species have the natural ability to utilize oligosaccharides (Gong et al. 2014) which can improve the fermentability of substrate further. Therefore, utilization of LCB hydrolysates containing mixture of sugars as well as various forms of saccharides must be carefully addressed in fermentation for achieving co-utilization as well as avoiding diauxic growth effects. Screening of suitable substrates and microbial species for substrate microbial compatibility must become an essential pre-fermentation step to implement an optimal substrate-microorganism combination during fermentation.

14.8.3 Fermentation Conditions

Overaccumulation of lipids never occurs under normal physiological conditions in microorganisms. In oleaginous microorganisms, substantially high carbon to nitrogen ratio can maintain the progression of lipid accumulation triggered mainly by nitrogen limitation. For attaining highest lipid content (amount of lipid per gram dry cell weight) and titer (concentration of lipid produced per liter volume), molar C/N typically higher than 65 and up to 100 (Jin et al. 2015) and w/w C/N higher than 50 up to 150 (Diwan and Gupta 2018a) are optimum. Optimal temperature for growth ranges from 25 to 30 °C and 20 to 28 °C, while optimal pH are usually 4–7 for oleaginous yeasts and molds, respectively (Jin et al. 2015). Lipid accumulation typically occurs within a 3 °C window of growth temperature while variation in pH from growth range does not significantly affect lipid production. Since nitrogen is an essential macromolecule for microbial growth, such depletion not only increases the lipid synthesis but at the same time imposes negative effect on biomass

accumulation which in turn diminishes the overall lipid production. Therefore, alternate of nitrogen, i.e., phosphorus, and sulfur limitation for triggering lipid synthesis are now being explored (Wu et al. 2010, 2011). Since oleaginicity is purely a stress mediated metabolic process, it can vary with the oleaginous species, substrate in question as well as cultivation conditions applied, which must be customarily optimized. Maintaining an optimal condition for achieving a balanced biomass and lipid concentration is the key to successful SCO fermentation strategy. Some emerging simple cultivation methods like open ponds (Santamauro et al. 2014) can also be explored as substitute to traditional fermentation.

14.8.4 Recovery of Oils

Recovery is a very essential step in SCO fermentation which decides the overall lipid yield after the bioprocess. The typical scheme of oil recovery includes cell harvesting followed by lipid extraction either from dried cell mass or wet biomass. Coagulation or flocculation, centrifugation and filtration are the usual cell harvesting techniques (Ahmad et al. 2014) which can elevate the cost of downstream processing in case of low cell density fermentations. The next step after harvesting is cell drying, which is more desirable than direct lysis of wet biomass, because dried biomass has been associated with better lipid yield during extraction (Kim et al. 2013). Since drying can be energy demanding and an economic liability, several other methods have been explored to improve the efficiency of wet biomass disruption like acid or enzymatic hydrolysis, bead beating, high-pressure homogenization, microwave treatment, and ultrasonication (de Boer et al. 2012; Jin et al. 2012; Kim et al. 2013). The most successful and universally implemented lipid extraction methods are the Bligh and Dyer and the Soxhlet method. Majority of the conventional lipid extraction strategies are either the basic Bligh and Dyer protocol or its modified variant. The principle of these methods is based on aqueous two phase extractions using organic solvents like chloroform, methanol, or hexane and water acting as aqueous phase. In these methods, although the extraction is efficient, solvent recovery is relatively inefficient and energy demanding.

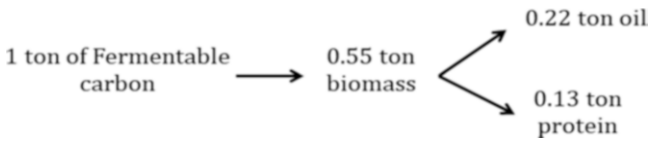
To overcome this, use of other alternatives like supercritical liquids have been assessed in lipid extraction (de Boer et al. 2012; Kim et al. 2013). Application of SCO also governs the fate of lipid recovery, like if they are intended for biodiesel conversion, the lipids are many a times extracted and transesterified simultaneously (de Boer et al. 2012; Zhang et al. 2014). Another efficient and promising energy extraction technique is HTL (discussed earlier) which is currently applied to bio-oil extraction from algal biomass. Some other such extraction processes include direct combustion, gasification and pyrolysis (Milledge and Heaven 2014). These processes can have positive implication in lignocellulosic lipid biorefineries as they lead to simultaneous generation of valuable coproducts and hence leverage the process economy.

14.9 The Biorefinery Concept for Positive Economics

Even though inexpensive LCB can assist in minimizing the process cost, the inefficient microbial carbon to lipid conversion economics will still make the practicability of this product contentious. The discussion thus far has already thrown light on how many a times the process of biomass to lipid and lipid to fuel or other product conversion coproduces other valuable commodity chemicals. Also the byproducts and leftover residues can be recycled or reused for value addition. These strategies can support in maintaining positive SCO economics.

14.9.1 SCO and Multiple Coproducts

Strategies like simultaneous saccharification and fermentation, seen in case of fermentative products such as ethanol and hydrogen, can merge the two separate steps of substrate preparation and fermentation potentially leading to reduction in process cost and complexity. However, the cellulolytic enzymes needed to saccharify lignocelluloses are usually secreted in the temperature range that is higher than that is required for lipid accumulation. Additionally, it is rare to find yeasts or fungi displaying both cellulolytic and oleaginous properties simultaneously. In 2012, Chinese physicist Zheng in his work reported a thermophilic fungus *T. lanuginosus* which displayed oleaginous property at 50 °C and converted wheat straw hydrolysate into lipids (Zheng et al. 2012). Finding and engineering such thermotolerant oleaginous species for producing cellulolytic enzymes can realize the simultaneous saccharification and fermentation strategy. Alternatively, oleaginous strains could be modified for production of hydrolytic enzymes, which will saccharify LCB, and released fermentable sugars will be capitalized in lipid synthesis. Ren et al., in 2015, showed synchronous production of two valuable products of lipid and hydrogen which can be an attractive coproduction scheme (Ren et al. 2015). In the similar way, oleaginous strains can be improved for coproducing other contemporary valuable metabolites also. Another interesting product is the defatted biomass left after lipid removal, which becomes a protein-rich substitute termed as single cell protein (SCP) with high value in nutrition and cattle feeding industry. As per Boulton 1988.



It is already known so far that 150 tons of fermentable carbon will produce 30 tons of oil/day, the minimum feasible per day requirement of oil. Biomass produced in this process will be around 80 tons and defatted biomass will amount to around 20 ton protein. A significant quantity of SCP is being produced in process, which

will add value and leverage the production cost. Some other valuable metabolites like amylase, carotenoids, and polygalacturonase (Papanikolaou et al. 2007, 2003; Saenge et al. 2011) have also reportedly been coproduced with SCO using agro-wastes. Several factors, especially the strains and the fermentation conditions, need critical assessment in the realistic scale-up study of these integrative and coproduction strategies.

14.9.2 Recycle and Reuse of Byproducts

Fermentative bioprocess from upstream to downstream processing involves numerous steps. When the bioprocess is associated with LC feedstocks, the number of steps shoots even higher. All these steps side by side release large amounts of byproducts which are usually refused without any processing and lead to environmental and ecological distress. These residues have high potential to be capitalized into other valuable products which can not only reduce environmental hazards but also curb the overall expenses. The defatted biomass apart from serving as SCP can also serve as organic fertilizer substitute and animal feed (Ryu et al. 2013) or dumps for composting. Upon processing, they can be converted into valuable amino acids, peptides, bioplastics, biomaterials, and biofoam (Jin et al. 2015) with wide commercial applicability. Bioprocess like SCO production releases large amount of spent broths and residual biomass. In a lignocellulosic fermentation, the bioprocess starts with biomass pretreatment which generates lignin, which is usually discarded. This byproduct has gained immense importance in the past few years not only for production of valuable commodity chemicals but also as a substrate for SCO itself. Studies have shown that lignin-rich organosolv pretreatment effluents of pine when used as substrate, actinomycetes *Rhodococcus opacus*, displayed 27% lipid content. Ethanol organosolvent digested lignin, ultasonicated lignin, as well as lignin generated model compounds have also been reported to serve as substrate for lipid production by *Rhodococcus* strains (Kosa and Ragauskas 2013). Currently, researches are also ongoing on use of lignin in microbial fuel cells, which can produce electricity and simultaneously generate various lignin-derived valuable chemicals such as vanillin, xylene, toluene, benzoic acid, and various phenols. Next major refuse generated in fermentation industries are effluent broths. The bioprocesses are water intensive and discharge huge amounts of high biochemical oxygen demand (BOD) effluents which are contaminating the existing water bodies. These effluents are loaded in various lignocellulosic degradation products and fermentation byproducts like acetic acid, anthocyanins, caramel, furfurals, HMF, melanoidins, tannins, and other secondary metabolites and toxins. High BOD and chemical oxygen demand (COD) effluents cause eutrophication and oxygen reduction consequently leading to depletion of aquatic organisms. Since freshwater is an essential resource, careful water utilization during fermentation and management of generated broths are crucial. If the bioprocess is releasing a low BOD and COD broths with minimal amounts of byproducts, the broths can be used for recycling. This can lead to

reduction in volumetric discharge and increase in substrate to product conversion during consecutive broth recycles, especially in case of high nutrient (Carbon) demanding fermentation like SCO. Highly contaminated broths can be mildly treated and replenished with macro and micro nutrients before recycling. American scientist Hsiao applied this concept practically for SCO production and carried out 75% of synthetic fermentation broth recycling seven times. When whey was used as substrate drop in lipid production was observed after three 50% recycle ratios (Hsiao et al. 1994). In a recent research, attempts were driven to recycle 100% fermentation broth consisting of LCB-rice straw hydrolysate as C source for MCFA-rich SCO production via yeasts *C. tropicalis* and *P. kudriavzevii*. The efforts led to increase in ratio of product to effluent generation (Diwan and Gupta 2018b).

However, if high BOD/COD effluents are being refused, different treatment protocols must be followed before discharging which can lower their pollution quotient and at the same time extract value from it. Many fermentation industries including distilleries are nowadays adapting such system. Effluents need to be digested through anaerobic digester units. The produced bio-methane has its own value or can be oxidized to methanol or converted to other energy forms. Released effluent gases can be capitalized for several other industrial processes. Leftover refuse can be used for bio-composting. Sometimes even after the range of aforementioned treatments, the BOD level did not drop significantly due to presence of salts or leftover organic matters. These residual effluents can be concentrated via evaporation followed by incineration which can be utilized for power production. Few organizations are already working on biorefinery concept and hence are reutilizing wastes for value addition like the USA based Industrial Microbes, which is producing commodity chemicals utilizing the methane and released effluent gases. Recycle and reuse of generated byproducts can create a lot of difference from economical, ecological, and environmental point of view. Such clean practices must be engrained and embraced to practically realize the biorefinery concept. Figure 14.4 shows an overview of the entire concept of lignocellulosic biomass to SCO from the circular biorefinery viewpoint.

14.10 Conclusion and Future Prospects

Conversion of LCB to SCO is not a simple single step process; it includes chain of processes each engendering different sets of challenges. After years of research and development, it is still believed that SCO is potentially going to become an important biorefinery product. According to the estimated statistics reviewed by Diwan et al. 2018, use of lignocellulosic feedstocks can reduce the cost expectancy of the SCO significantly compared to synthetic sugar based fermentations. With endless debate and ambiguity, it is difficult to predict the fate of SCO. In any of the case SCO currently seems more economically viable in production of low volume- high value oils either using synthetic substrates or by replacing them with edible agricultural biomasses. Even though cost becomes a secondary criterion when product has

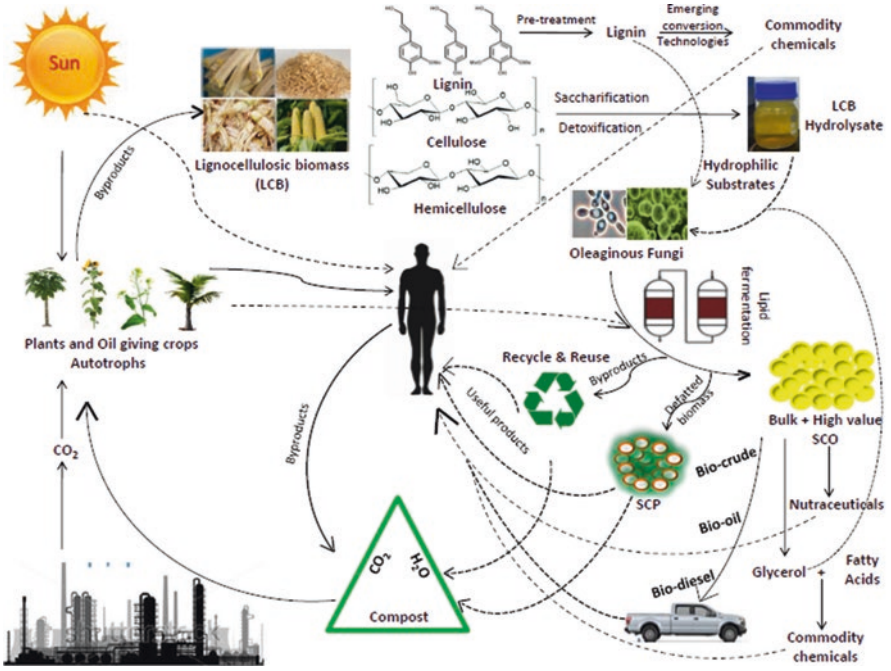


Fig. 14.4 Lignocellulosic biomass to SCO: a circular biorefinery

nutraceutical importance and intended for consumption, use of edible agro-byproducts can curb the expenses to a bit. Oils pursuing conversion to biofuels needs bulk production while low lipid yield and titer is severely impeding this possibility and their economic feasibility. Strain improvement for achieving high lipid yield, high tolerance to degradation products and coproduction of other valuable metabolites, refinement in LCB pretreatment and saccharification technology, improvement in process design and fermentation conditions need to be addressed. The realistic strategy which can have practical feasibility is balanced coproduction of neutral bulk lipids (which can be pursued for biofuels) with functional fatty acids (which can increase the economic value of these oils and to some extent balance the overall expense). Co-generation of valuable products and value addition to byproducts can prove to be the backbone for the techno-economics of SCO production and hence must also be well engrained in the bioprocess.

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Chapter 15
Recent Advancement and the Way Forward for *Cordyceps*

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15.1 Introduction

Cordyceps species are entomopathogenic fungi belonging to the division *Ascomycota*, class *Sordariomycetes*, orders *Entomophthorales* and *Hypocreales*. So far, reported species were grouped under 91 genera. Recently, this fungal study has become a very important topic because of their strong medicinal properties and huge global markets. They are excellent biocontrol agent attacking larvae and pupae (Kobayasi 1941, 1982). Fungi/mushrooms have been used by humans since thousands of years as food, food supplements, and/or traditional medicine. More than 14,000 species of mushrooms are recognized, and among them, approximately 2000 are identified as edible (Vikineswary et al. 2013). Fungi form the second largest group after insects, and it is believed that 1.5 million fungi exist in nature (Mueller et al. 2011; Chiu et al. 2016). They have attracted researchers from different disciplines owing to their fascinating nature and capability to survive in antagonistic environments and the midst of decay at the toughest layer of the ecosystem (Lu et al. 2013; Chiu et al. 2016). Entomopathogenic fungi produce a number of secondary metabolites during their infection and proliferation in insects. To human beings, these secondary metabolites served as biofunctional agents which were evolved over centuries with amazing potential in improving health and preventing diseases (Lu et al. 2013; Chiu et al. 2016).

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Many natural *Cordyceps* sp. are used in traditional Chinese medicines in China, Japan, Korea, Taiwan, and other eastern Asian countries. Yarsa gumba is known to science as *Ophiocordyceps sinensis*. In Chinese, it is called *Dong cong xia cao*. However, the origins are Tibetan: *Yart Swa Gun Bu*, which means "herb in the summer and insect in the winter." This fungus is also named as "Keera Jhar" (insect herb)/Keeda ghass/Jeevan buti/Chyou kira/Sanjeevani buti or Himalayan Viagra by local peoples in Indian mountains (Panda and Swain 2011). In addition, *Cordyceps militaris*, a type species of cordyceps, has been regarded as substitute for "*Dong cong xia cao*" and is named as "*North dong-chong xia cao*" (Lo et al. 2013).

Entomopathogenic fungi may be poly- and pleomorphic. Their life cycle may contain a meiotic (teleomorphic, prefect) stage and many mitotic (anamorphic, imprefect) stages. Both the teleomorph and anamorph may have different morphology (Kobayasi 1941; Hodge et al. 1998). These fungi produce a stroma (fruiting body) on infected arthropod. The color of the stroma depends on the species, and produces a stipe, which is buried in the soil or dead trees. The fertile region of the stroma is terminally positioned and has a head-like appearance. Spores produced through sexual reproduction inside sacs, are housed in perithecia. Ascospores and asci are microscopic structures; a single perithecium, which gives the stroma a small blade-like shape (Hodge et al. 1996; Kobayasi and Shimizu 1960; Liang et al. 1991; Liu et al. 2001; Evans et al. 2011). Infection on the surface of insect body during winter leads to the formation of a fruiting body in summer followed by the spores becoming airborne. The elongated stroma look like cylindrical or branched and is often found bursting from the head of the host (Fig. 15.1). The fruiting body, bears many infectious perithecia containing ascospores. Many *Cordyceps* species can grow on artificial media, and some can be isolated from soil (Zhu et al. 1998; Winkler 2008, 2010).

Fig. 15.1 *Cordyceps sinensis* in its natural habitat (4550 meters in Tibet, China). (Sources: Holliday and Cleaver 2008)

These fungi attack hosts from many orders of Lepidoptera (Humber 2000; Karpinska 2012; Hardeep et al. 2014), usually that of the Himalayan ghost moth larvae *Thitarodes armoricanus* (=*Hepialus armoricanus*). The insect may infected at various life stages i.e. larvae, pupae and adult. Infection starts with the dispersion of fungal conidia on insect cuticle. The spores then adhere to the outer surface of insect body, and germinate within a few hours (Hardeep et al. 2014). Conidia secreats protective enzymes (superoxide dismutase and peroxidases) and hydrolytic enzymes (proteases, chitinases, and lipases) to protects the fungi during germination (Isaka et al. 2001; Wang et al. 2005; Holder and Keyhani 2005; Hardeep et al. 2014; Kaszak 2014). The conidia produces a germ tube with an appressorium (a flattened disk-like structure on the end). The appressorium penetrates the exoskeleton and enter the hemocoel by a combinational effect of mechanical pressure and enzymes. During development and growth fungus produces insecticidal secondary metabolite in side the host body resulting death of the host. The fungal hyphae then feed on the insect, growing throughout all visceral organs. Finally, the tissue of the host is replaced with a fungal mycelium, and only the host exocuticula is avilable for supports of the fungal stroma (Hodge et al. 1996; Isaka et al. 2001; Wang et al. 2005; Holder and Keyhani 2005; Hardeep et al. 2014; Kaszak 2014).

15.2 History

Gordon Wasson (father of modern ethnomycology) believed that the Soma plant used in religious ceremonies, over 4000 years ago, by the "Aryans" was a mushroom. The Vedic juice called "soma rasa" is said to provides divine qualities to the consumer soul, even immortality. As per Ayurveda mushrooms classified as tamasika ahara i.e. a medicine for enhancing vigor and vitality (Panda and Swain 2011). This fungus has been known and used as a medication in China for over 400 years (Wasson 1968; Adhikari 1981; Hodge et al. 1998; Holliday and Cleaver 2008; Panda and Swain 2011; Lu et al. 2013). The earliest record outlining the tonic properties especially as an aphrodisiac is a fifteenth-century Tibetan medical. It was initially recorded in Ben-Cao-Bei-Yao by Wang Ang in 1694. This fungus was first introduced to Western society during the seventeenth century, and its uses were documented in the Qing dynasty Bencao Congxin (New Compilation of Materia Medica) in 1757. In 1878 Saccardo, an Italian scholar, named *Cordyceps* derived from Latin words *cord* and *ceps*, respectively, meaning "club" and "head." The Latin word conjunction accurately describes the appearance of these club fungi, whose stroma and fruit body extend from mummified carcasses of insect larvae, and this nomenclature has been adopted up to the present day (Holliday and Cleaver 2008).

The fungus was made famous in 1993 by the performance of three female Chinese athletes who broke five world records for long-distance running. They consumed *Ophiocordyceps sinensis* and turtle blood (Winkler 2008; Wang et al. 2008; Hardeep et al. 2014; Kaszak 2014). In 2006, the demand of wild *Cordyceps sinensis*

was increased and the price of the this "soft gold" reached up to $32,000/kg in China (Au et al. 2012; Chiu et al. 2016). The collection of this fungi was difficult and not able to fulfill the incresed demands. Thus researchers concentarted for developing fermentation technologies to harvest large amounts of biomass for functional foods (Shih et al. 2007; Chiu et al. 2016). Functional foods can serve health benefits and nutritional security to an effected population. These kinds of foods, which can protect or delay the onset of chronic diseases (cancer, diabetes mellitus, and cardiovascular and obesity diseases etc.), become a necessity rather than luxury (Granato et al. 2010; Chiu et al. 2016).

15.3 Distribution

The distribution of these forest fungi is cosmopolitan (Li et al. 2011; Shrestha et al. 2012, 2016). Entomophthoralean fungi generally show narrow host range and are distributed in temperate forests and hardly reports from tropical regions. While hypocrealean (cordyceps), have narrow to very broad host range and are dominantly distributed in humid tropical forests (Burges 1981; Evans 1982; Kobayasi 1982; Li et al. 2011; Vega et al. 2012; Kaszak 2014). The *Cordyceps* species are highly diverse in subtropical and tropical regions with a hot and humid climate i.e. Asia. A total of 203 localities have been found, of which 106 are considered as confirmed distribution sites, 65 as possible distribution sites, and 29 as excluded distribution sites (Li et al. 2011) (Fig. 15.2). Li et al. (2011) reported that *Cordyceps* is confined to the Tibetan Plateau regions, including Gansu, Qinghai, Sichuan, Tibet and Yunnan provinces in China and in certain areas of the southern flank of the Himalayas, in the countries of Bhutan, India, and Nepal, with 3000 m (Kobayasi 1980; Shen et al. 1980; Xiao et al. 1983; Yin et al. 1990; Negi et al. 2009).

Fig. 15.2 Worldwide distribution of *Cordyceps sinensis.* (Sources: Li et al. 2011)

The fungus is distributed from the southernmost site in Yulong Naxi in northwestern Yunnan Province to the northernmost site in the Qilian Mountains in Qinghai Province, and from the east edge of the Tibetan Plateau in Gansu Province, to the western most site in Uttarakhand, India (Li et al. 2011). This fungus is found in extensive quantity in Api in Dharchula, Baling, Bon, Budhi, Chipla, Chal, Dugtu, Galja, Karschila, Malpa top, Nampa, Njyang top, Panchachuli and Pithoragarh (Garbyal et al. 2004).

15.3.1 Type

The entomopathogenic fungi mainly belong to two diverse groups of kingdom *Fungi*, *Entomophthorales* (phylum *Entomophthoromyota*) and *Hypocreales* (cordyceps) (phylum *Ascomycota*). These fungi are distributed in various terrestrial ecosystems including Arctic Circle and Antarctica (Karam and Karam 2012; Shrestha et al. 2016). Before, Sung et al. (2007), *Cordyceps* was placed in family *Clavicipitaceae* of order *Hypocreales*. The phylogenetic studies showed that both *Cordyceps* and *Clavicipitaceae* were not monophyletic (Artjariyasripong et al. 2001; Stensrud et al. 2005; Spatafora et al. 2007; Sung et al. 2007). The *Cordyceps* was segregated into diffrent phylogenetic genera within three families of *Hypocreales* (Sung et al. 2007). According to the phylogenetic classification, *Cordyceps* is now restricted to the clade containing the type species *C. militaris*, circumscribed to *Cordycipitaceae* (Sung et al. 2007). Newly segregated genera *Ophiocordyceps* and *Elaphocordyceps* were placed under family *Ophiocordycipitaceae*; the other two genera *Metacordyceps* and *Tyrannicordyceps* remained in *Clavicipitaceae* (Sung et al. 2007; Kepler et al. 2012). Following the recent revision in the International Code of Nomenclature for algae, fungi, and plants (ICN), *Elaphocordyceps* is now synonymized with *Tolypocladium* (Quandt et al. 2014) and *Metacordyceps* with *Metarhizium* (Kepler et al. 2014).

15.3.2 Host Range and Species Affinity

The host range of *Cordyceps* is very wide and includes several orders, viz., Araneae, Blattodea, Coleoptera, Dermaptera, Diptera, Hemiptera, Hymenoptera,Lepidoptera, Mantodea, Odonata, Orthoptera, Phasmatodea, etc. (Evans 1982; Wang and Yao 2011; Shrestha et al. 2012, 2016). Nearly 60% of the *Cordyceps* species are classified under two orders Coleoptera and Lepidoptera (Shrestha et al. 2012, 2016). The majority of hosts (>95%) in Lepidoptera (moths and butterflies) and Coleoptera (beetle) are larvae with very few adults or pupae, making the host identification more difficult. In contrast, majority of hosts in other orders are adults such as spider (Araneae); cockroach and termite (Blattodea); fly (Diptera); earwig (Dermaptera); cicada, bug, scale insect, and coccid (Hemiptera); ant, bee, and wasp

(Hymenoptera); grasshopper, mantis (Mantodea); dragonfly (Odonata); locust, and cricket (Orthoptera); and stick insect (Phasmatodea) (Evans 1982). However, Why lepidopterans and coleopterans orders are mostly susceptible at larval stage and other orders are more susceptible at adult stage is not well understood (Shrestha et al. 2016).

Lepidoptera is one of the largest orders of insects (Gaston 1991) with 160,000 spp. classified into 4 suborders, 45 superfamilies, and 139 families (Nieukerken et al. 2011). Out of four suborders (Aglossata, Glossata, Heterobathmiina, and Zeugloptera), *Cordyceps* and allied genera are known only from Glossata. It is the largest suborder consisting of almost 99.9% of all described lepidopterans (Nieukerken et al. 2011; Wagner 2001). It is further classified into six infraorders (Dacnonypha, Acanthoctesia, Lophocoronina, Neopseustina, Exoporia, and Heteroneura) (Nieukerken et al. 2011; Wagner 2001). Among them, *Cordyceps* hosts are parasitizing only two infraorders Exoporia and Heteroneura. Exoporia is a small infraorder consisting of 2 superfamilies (Hepialoidea and Mnesarchaeoidea) with 636 named species worldwide (Shrestha et al. 2016). The superfamily Hepialoidea comprises five families (Anomosetidae, Neotheoridae, Prototheoridae, Palaeosetidae, and Hepialidae) distributed in diverse vegetation of which *Cordyceps* species are recorded from the family Hepialidae only (Table 15.1). The infraorder Heteroneura consists of more than 98% of lepidopteran species with more than 30 superfamilies (Nieukerken et al. 2011; Wagner 2001), of which 9 superfamilies (Noctuoidea, Zygaenoidea, Cossoidea, Drepanoidea, Tineoidea, Papilionoidea, Geometroidea, Bombycoidea, and Lasiocampoidea) are recorded as hosts of *Cordyceps* spp. worldwide. Among the host families, Tineidae is the only micro-lepidopteran family, and the rests are macrolepidopterans. Papilionidae and Pieridae are the two families of butterfly and all others being moths (Table 15.1).

Coleoptera is currently the most species-rich group on this planet (Slipinski et al. 2011). The order is classified into four suborders (Adephaga, Archostemata, Myxo phaga, and Polyphaga) (Bocak et al. 2014). Polyphaga is the largest suborder (>170 families) covering 90% of total beetle species. Adephaga is the second largest suborder, followed by Myxophaga and Archostemata (Bocak et al. 2014). All the known coleopteran hosts belong to suborders Polyphaga and Adephaga under 8 superfamilies (Scarabaeoidea, Elatroidea, Chrysomeloidea, Cucujoidea, Curculionoidea, Tenebrionoidea, Staphylinoidea, and Caraboidea) and 11 families (Scarabaeidae, Geotrupidae, Lucanidae, Elateridae, Cerambycidae, Chrysomelidae, Erotylidae, Curculionidae, Tenebrionidae, Staphylinidae, and Crambidae) (Nieukerken et al. 2011; Wagner 2001; Shrestha et al. 2016) that are listed in Table 15.1.

15.4 Medicinal Properties and Their Uses

Species of *Cordyceps* have long been known and used to promote longevity, relieve fatigue, and treat numerous diseases in traditional Chinese medicine (Hobbs 1995; Russell and Paterson 2008). Perusal of literature showed that cordyceps has a wide

Table 15.1 List of *Cordyceps* species recorded on various hosts

Host family	Host genus/species	Host stages	*Cordyceps* sp.	References
Hepialidae	*Oxycanus dirempta*, *Abantiades* sp.	Larva	*C. cranstounii*	Willis (1959)
	—	Larva	*C. cuncunae*	Palfner et al. (2012)
	Oxycanus sp., *Trictena* sp., *Abantiades* sp.	Larva	*C. hawkesii*	Olliff (1895), Willis (1959)
	Endoclita excrescens	Larva	*C. hepialidicola*	Lim et al. (2001)
	Thitarodes armoricanus	Larva	*C. kurijimeansis*	Negi et al. (2012)
	Hepialus sp.	Larva, pupa	*C. militaris*	Petch (1948)
	Abantiades labyrinthicus, *Aoraia enysii*, *Oxycanus* sp., *Trictena atripalpis*	Larva, pupa	*D. gunnii*	Olliff (1895), Glare et al. (1993), Willis (1959)
	Thitarodes baimaensis	Larva	*O. crassispora*	Zang et al. (1990)
	—	Larva	*O. emeiensis*	Liu and Liu (1997)
	Ahamus altaicola, *Hepialus humuli*, *Korscheltellus lupulina*, *Parahepialus nebulosus*	Larva	*O. gracilis*	Zang and Kinjo (1998), Lauritzen (1971)
	Aenetus virescens, *Aoraia dinodes*, *A. ensyii*, *Wiseana* spp., *Ahamus anomopterus*, *A. gangcaensis*, *A. jianchuanensis*, *A. lijiangensis*, *A. luquensis*, *A. maquensis*, *A. sichuanus*, *A. yulongensis*, *A. yunlongensis*, *A. yunnanensis*, *A. yushuensis*, *A. zadoiensis*, *A. zhayuensis*, *Bipectilus yunnanensis*, *Endoclita davidi*, *Gazoryctra ganna*, *Hepialus xiaojinensis*, *Magnificus jiuzhiensis*, *M. zhiduoensis*, *Parahepialus nebulosus*, *Pharmacis carna*	Larva	*O. robertsii*	Glare et al. (1993), Berkeley (1855), Miller (1952)

(continued)

Table 15.1 (continued)

Host family	Host genus/species	Host stages	*Cordyceps* sp.	References
	P. fusconebulosa, *P. pyrenaicus*, *Thitarodes albipictus*, *T. armoricanus*, *T. baimaensis*, *T. baqingensis*, *T. bibelteus*, *T. biruensis*, *T. callinivalis*, *T. cingulatus*, *T. damxungensis*, *T. deqinensis*, *T. dongyuensis*, *T. ferrugineus*, *T. gonggaensis*, *T. jialangensis*, *T. jinshaensis*, *T. kangdingensis*, *T. kangdingroides*, *T. latitegumenus*, *T. litangensis*, *T. markamensis*, *T. meiliensis*, *T. namensis*, *T. namlinensis*, *T. oblifurcus*, *T. pratensis*, *T. pui*, *T. renzhiensis*, *T. varians*, *T. xunhuaensis*, *T. yeriensis*, *T. zaliensis*, *T. zhongzhiensis*	Larva	*O. sinensis*	Zou et al. (2010), Wang and Yao (2011)
	Trictena sp.	Pupa	*O. taylorii*	Willis (1959)
	Ahamus jianchuanensis, *A. yunnanensis*	Larva	*O. lanpingensis*	Chen et al. (2013)
	Ahamus yunnanensis	Larva	*O. laojunshanensis*	Chen et al. (2011a, b)
	Endoclita nodus	Larva	*O. ramosissimum*, *O. xuefengensis*	Wen et al. (2014), Wen et al. (2013)
Noctuidae	*Panolis flammea*, *Euxoa ochrogaster*, *Colocasia coryli*, *Arcte coerula*, *Dasypodia selenophora*	Larva	*C. alpicola* *C. militaris*	Kobayasi and Shimizu (1976a, b), Kobayasi (1941)
	Acronicta americana	Larva, pupa	*O. elongate*	Petch (1937)
	—	Pupa	*C. bifusispora*	Eriksson (1982)
	—	Larva	*C. bulolensis*	Kobayasi and Shimizu (1976a, b)
	—	Adult	*C. cristata*	Moller (1901)
Sphingidae	—	Pupa	*C. flavobrunnescens*	Kobayasi (1941)

(continued)

Table 15.1 (continued)

Host family	Host genus/species	Host stages	*Cordyceps* sp.	References
	Clanis bilineata	Larva	*C. kyusyuensis* *C. taishanensis*	Kawamura (1955), Liu et al. (1984)
	Sphinx pinastri, *Hyles euphorbiae*, *Mimas tiliae*, *Marumba sperchius*, *Laothoe populi*, *Callambulyx tatarinovii*	Larva, Pupa	*C. militaris*	Kryukov et al. (2011), Bary (1867), Kobayasi (1941), Gu and Liang (1987), Panigrahi (1995), Chen (1997), Sopp (1911), Gray (1858), Sato et al. (1994), Hitchcock (1961)
	—	Larva, pupa	*C. polyarthra*	Moller (1901)
	Amphipyra effusa, *Amphonyx duponchel*, *A. jatrophae*, *Anceryx ello*, *Cocytius* sp., *Macroglossum insipida*, *Sphinx pinastri*	Adult	*C. tuberculata*	Kobayasi (1941), Petch (1934), Maire (1917)
Saturniidae	*Actias artemis* *Anisota senatoria*	Larva, Pupa	*C. longdongensis* *C. militaris*	Liu (1997), Kobayasi (1941)
Bombycidae	*Bombyx* sp., *Bombyx mori*	Larva, Pupa	*C. michaelisii* *C. militaris*	Hennings (1902), Kobayasi (1941)
Endromidae	*Andraca bipunctata*	Larva	*C. militaris*	Kryukov et al. (2011), Bary (1867), Kobayasi (1941), Gu and Liang (1987), Panigrahi (1995), Chen (1997), Sopp (1911), Gray (1858), Sato et al. (1994), Hitchcock (1961), Pacioni and Rossi (1980)
Erebidae	*Calliteara pudibunda*, *Leucoma salicis*	Larva	*C. militaris*	
	—	Larva	*C. nikkoensis*	
Drepanidae	*Achlya flavicornis*, *Ochropacha duplaris*, *Tethea ocularis*, *Tetheella fluctuosa*	Larva	*C. militaris*	
Geometridae	*Biston panterinaria*, *Lycia hirtaria*	Larva	*C. militaris*	
Geometridae	*Triphosa* sp.	Adult	*C. riverae*	
Lasiocampidae	*Dendrolimus pini*, *D. superans*, *Macrothylacia rubi*	Larva	*C. militaris*	
Notodontidae	*Fentonia ocypete*, *Lampronadata cristata*, *Phalera assimilis*, *P. bucephala*, *Syntypistis punctatella*	Larva	*C. militaris*	
Tineidae	—	Larva	*C. cardinalis*	Sung and Spatafora (2004)

(continued)

Table 15.1 (continued)

Host family	Host genus/species	Host stages	*Cordyceps* sp.	References
Papilionidae	—	Adult	*C. tuberculata*	Kobayasi (1941), Petch (1934), Maire (1917)
Pieridae	*Pieris rapae*	Larva	*M. taii*	Zang and Kinjo (1998)
Limacodidae	—	Larva, pupa	*O. cochlidiicola*	Kobayasi and Shimizu (1980)
Cossidae	*Yakudza vicarius*	Larva	*M. indigoticum*	Kobayasi and Shimizu (1978)
	Cossus sp.	Larva	*O. arborescens* *O. macroacicularis* *C. bassiana*	Ban et al. (2015),
Scarabaeidae	*Heteronyx* sp.	Larva	*C. brittlebankii*	McLennan and Cookson (1926)
	Anomala cuprea	Larva	*C. brongniartii*	Shimazu et al. (1988)
	Lepidiota sp.	Larva	*C. coxii*	Olliff (1895)
	Aphodius howitti, *A. tasmaniae*	Larva	*O. aphodii*	Mathieson (1949), Glare et al. (1993)
	—	Larva	*O. arbuscula*	Teng (1936)
	—	Larva	*O. barnesii*	Massee (1895)
	—	Larva	*C. obliquiordinata*	Kobayasi and Shimizu (1982)
	Melolontha sp.	Larva	*C. pseudoinsignis*	Moureau (1949)
	—	Adult	*C. scarabaeicola*	Kobayasi and Shimizu (1976a, b)
	—	Larva	*M. brittlebankisoides*	Liu et al. (2001)
	—	Larva	*O. geniculata*	Yahagi (2008)
	—	Larva	*O. gracillima*	Sanjuan et al. (2015)
	—	Larva	*O. highlandensis*	Yang et al. (2015)
	—	Larva	*O. macularis*	Kobayasi (1977)
	Ancyloncha puncticollis, *Lachnosterna fusca*, *Melolontha* sp.	Larva	*O. melolonthae*	Massee (1895), Ellis and Everhart (1892)
	—	Larva	*O. neovolkiana*	Kobayasi (1941)
	—	Larva	*O. nigrella*	Kobayasi and Shimizu (1983)
	Lachnosterna fusca, *Phyllophaga* sp., *Rhizotrogus* sp.	Larva	*O. ravenelii*	Ellis and Everhart (1892), Berkeley (1857), Mains (1958)
	—	Larva	*S. palustris*	Moller (1901)
	Scarabaeus sp.	Larva	*O. michiganensis*	Kobayasi (1941), Roth and Clerc (1997)

(continued)

Table 15.1 (continued)

Host family	Host genus/species	Host stages	*Cordyceps* sp.	References
	Costelytra zealandica	Larva	*O. stylophora*	Kautman and Kautmanova (2009), Glare (1992), Moniz et al. (1999), MacMillan (1898)
Erotylidae	*Erotylus* sp.	Adult	*C. erotyli*	Petch (1937)
Geotrupidae	*Geotrupes* sp.	Adult	*C. geotrupis*	Teng (1934)
Elateridae	—	Larva	*C. huntii*	Massee (1899)
	—	Larva	*C. aurantiaca*	Keissler and Lohwag (1937)
	Melanotus communis	Larva	*C. nirtolii*	Negi et al. (2012)
	—	Larva	*C. rubra*	Moller (1901)
	Melanotus caudex, *Pleonomus canaliculatus*	Larva	*C. shanxiensis*	Liu et al. (1985)
	Melolontha sp.	Larva	*C. velutipes*	Moureau (1949), Massee (1895)
	Campsosternus auratus	Larva	*M. campsosterni*	Zhang et al. (2004)
	Hemirhipus sp.	Larva	*M. martiale*	Spegazzini (1919)
	—	Larva	*O. brunneipunctata*	Hywel-Jones (1995)
	—	Larva	*O. elateridicola*	Kobayasi and Shimizu (1983)
	—	Larva	*O. gracilioides*	Yahagi (2008)
	Campsosternus auratus, *C. fruhstorferi*	Larva	*O. jiangxiensis*	Liang et al. (2001)
	—	Larva	*O. purpureostromata*	Kobayasi and Shimizu (1980)
	—	Adult	*O. salebrosa*	Mains (1958)
	Denticollis linearis	Larva	*O. stylophora*	Kautman and Kautmanova (2009), Glare (1992), Moniz et al. (1999), MacMillan (1898)
Staphylinidae	*Staphylinus* sp.	Adult	*C. memorabilis*	Cooke (1892)
	—	Larva	*C. staphylinidicola*	Kobayasi and Shimizu (1982)
Tenebrionidae	*Nictobates* sp.	Larva	*O. acicularis*	Massee (1895)
	—	Larva	*O. formosana*	Li (2002)
	Cylindronotus sp., *Helops caraboides*, *H. lanipes*	Larva	*O. larvicola*	Moureau (1949), Kobayasi (1941) Koval (1974)
Curculionidae	*Cryptorhynchus corticicolus*	Larva	*P. peltata*	Mains (1958)

(continued)

Table 15.1 (continued)

Host family	Host genus/species	Host stages	*Cordyceps* sp.	References
Tenebrionidae	*Tenebrio molitor*	Larva	*C. militaris*	Kryukov et al. (2011), de Bary (1867)
	Heilipus celsus	Adult	*O. curculionum*	Massee (1895)
Cerambycidae	*Oemona hirta*	Larva	*O. dovei*	Kobayasi (1941)
	Callidium sp.	Larva	*O. konnoana*	Yahagi (2008)
	Phoracantha semipunctata	Larva	*O. stylophora*	Kautman and Kautmanova (2009), Glare (1992), Moniz et al. (1999), MacMillan (1898)
Lucanidae	*Rhyssonotus nebulosus*	Larva	*O. scottiana*	Olliff (1895)
Carabidae	—	Larva	*C. nikkoensis*	Kobayasi and Shimizu (1983)
	—	Larva	*O. carabidicola*	Kobayasi and Shimizu (1980)
	Eripus heterogaster	Larva	*O. volkiana*	Moller (1901)
	Calathus sp., *Calosoma* sp., *Carabus auronitens*, *C. coriaceus*, *C. glabratus*, *C. hortensis*, *C. intricatus*, *C. nemoralis*, *C. nemorensis*, *C. violaceus*, *Coptolabrus* sp., *Hadrocarabus problematicus*, *Pterostichus* sp.,	Larva, adult	*O. elongatiperitheciata*	Kobayasi and Shimizu (1980), Massee (1895), Kobayasi (1951), Kautman and Kautmanova (2009), Kautmanova (2002), Klingen and Salinas (2002), Kobayasi (1937), Moingeon (2003), Zang and Kinjo (1998)
Cerambycidae Chrysomelidae Curculionidae Staphylinidae Tenebrionidae	*Leptura* sp., *Diabrotica* sp., *Apion flavipes, Ocypus* sp., *Meneristes laticollis*	Larva, adult	*O. entomorrhiza*	
Tipulidae	*Tipula paludosa*	Larva	*C. militaris*	Muller-Kogler (1965)
Cimbicidae	*Cimbex similis*	—	*C. militaris*	Kobayasi (1941)

Adopted and modified by Wang and Yao (2011) and Shrestha et al. (2012, 2016).

range of pharmacological properties, such as immunomodulating, anti-oxidant, anti-tumor, anti-cancer, anti-metastatic, anti-inflammatory, anti-oxidative, antibiotic, hepatoprotective, nephroprotective, hypoglycemic, and hypocholesterolemic effects (Park et al. 2005; Yoo et al. 2004; Lee et al. 2010; Ohta et al. 2007; Ahn et al. 2000; Wang et al. 2011a, b; Yue et al. 2013; Zhou et al. 2009; De Silva et al. 2012). These properties are due to the presence of a variety of bioactive compounds, which include polysaccharides, cordycepin, adenosine, sterol, ergosterol, protein and

amino acids, vitamins E and K, and the water-soluble vitamins B1, B2, and B12 (Zhou et al. 2009; De Silva et al. 2012). In addition, it also contains various sugars, including mono-, di-, and oligosaccharides, nucleosides, and macro- and microelements (K, Na, Ca, Mg, Fe, Cu, Mn, Zn, Pi, Se, Al, Si, Ni, Sr, Ti, Cr, Ga, V, and Zr) (Hobbs 1995; Holliday et al. 2004, 2005; Holliday and Cleaver 2008). More than 20 pharmacologically bioactive compounds and various extract using solvent (water, ethanol, methanol, and ethyl acetate) have been reported from mycelia, culture supernatants, and fruiting bodies of *cordyceps* that are listed in Table 15.2 (Yamaguchi et al. 2000; Koh et al. 2003; Ji et al. 2009; Lo et al. 2013; Chen et al. 2013; Chiu et al. 2016).

15.4.1 Polysaccharides

Polysaccharides that mainly play roles as energetic and structural components. Various forms of them have been identified such as extracellular polysaccharide, intracellular polysaccharide, exopolysaccharides, heteropolysaccharides, mannoglucan, D-glucan, etc. They are one of the major components of *Cordyceps* responsible for a large number of pharmacological properties, viz., immunomodulatory, anti-tumor, anti-metastatic, anti-inflammatory, anti-oxidative, hypoglycemic, hypolipidemic, and steroidogenic (Li et al. 2006a; Russell and Paterson 2008; Shashidhar et al. 2013).

15.4.2 Cordycepin

Cordycepin and cordycepic acid are the two important pharmacologically active compounds isolated from culture supernatant and fruiting bodies of *Cordyceps* (Huang et al. 2003). Cordycepin, which was originally extracted from *C. militaris*, is the main bioactive component of cordyceps (Kaczka et al. 1964; Cunningham et al. 1950), while cordycepic acid, an isomer of quinic acid, is subsequently identified as d-mannitol (Sprecher and Sprinson 1963; Jiang 1987). Cordycepin is now a days used in injections as a raw material and as a supplement in other medicines. Cordycepin helps in reducing the overall level of cholesterol, low-density lipoprotein, and triglycerides in the blood. Furthermore, its effect on glucose metabolism potentially regulates glucose level in the blood (Guo et al. 2010). Cordycepin also has anti-inflammatory and antitumor properties because it has the ability to impede RNA synthesis (Russell and Paterson 2008; Tuli et al. 2013).

Table 15.2 Pharmacologically bioactive compound/extract and its bioactivities and material source of *Cordyceps*

Bioactive compound/extract	Bioactivities	Material source	References
Compound			
Polysaccharides; extracellular	Immunomodulatory and anti-tumor	Culture supernatant	Song et al. (2012), Yoon et al. (2008), Zhang et al. (2005)
	Anti-oxidant	Culture supernatant	Cheung et al. (2009), Kuo et al. (2007a), Sheng et al. (2011), Wang et al. (2011a, b), Zhang et al. (2008)
Polysaccharides; intracellular	Immunostimulatory and anti-tumor	Mycelium	Leung et al. (2009), Yan et al. (2009)
	Immunomodulatory and anti-oxidant	Mycelium	Yan et al. (2011)
	Immunomodulatory	Mycelium	Chen et al. (2008)
	Hypoglycemic	Mycelium	Chen et al. (2010a), Chen et al. (2010b), Wu et al. (2006)
	Hypoglycemic and anti-oxidant	Mycelium	Huang et al. (2002), Kiho et al. (1993)
	Anti-oxidant and anti-tumor	Mycelium	Li et al. (2006a)
	Anti-oxidant	Mycelium	Chen et al. (2006)
	Anti-oxidant	Fruiting body	Wang et al. (2011a, b)
	Protection of chronic renal failure	Mycelium	Wang et al. (2009)
	Cholesterol esterase inhibitory activity	Mycelium	Wang et al. (2010)
	Lower plasma triglyceride and cholesterol	Mycelium	Kim (2010)
Cordycepin	Steroidogenesis	Culture supernatant	Kiho et al. (1996)
	Anti-metastatic activity	Culture supernatant	Leu et al. (2011), Pao et al. (2012)
	Anti-tumor	Culture supernatant	Kubo et al. (2012)
	Immunomodulatory	Culture supernatant	Chen et al. (2010b) Yoshikawa et al. (2007)
Adenosine	Immunomodulatory	Mycelium	Zhou et al. (2008)
Guanosine	Immunomodulatory	Mycelium	Yu et al. (2007)
Lovastatin	Hypolipidemic	Mycelium	Yu et al. (2007)
γ-Aminobutyric acid (GABA)	Neurotransmitter	Mycelium	Tsai et al. (2010)

(continued)

Table 15.2 (continued)

Bioactive compound/extract	Bioactivities	Material source	References
Sitosterol	Cytotoxic	Mycelium	Tsai et al. (2010)
Ergosterol	Cytotoxic	Mycelium	Matsuda et al. (2009)
5α,8α-Epidioxy-22E-ergosta-6, 22-dien-3β-ol	Cytotoxic	Mycelium	Matsuda et al. (2009)
5α,8α-Epidioxy-22E-ergosta-6, 9(11),22-trien-3β-ol	Cytotoxic	Mycelium	Matsuda et al. (2009)
5α,6α-Epoxy-5α-ergosta-7, 22-dien-3β-ol	Cytotoxic	Mycelium	Matsuda et al. (2009)
5α,8α-Epidioxy-24(R)-methylcholesta-6,22-dien-3β-D-glucopyranoside	Anti-tumor	Mycelium	Matsuda et al. (2009)
5,6-Epoxy-24(R)-methylcholesta-7,22-dien-3β-ol	Anti-tumor	Mycelium	Bok et al. (1999)
Serine protease	Fibrinolytic	Culture supernatant	Bok et al. (1999)
Melanin	Anti-oxidant	Mycelium	Li et al. (2007)
Cordysinin A	Anti-inflammatory	Mycelium	Lu et al. (2013)
Cordysinin B	Anti-inflammatory	Mycelium	Yang et al. (2011)
Cordysinin C	Anti-inflammatory	Mycelium	Yang et al. (2011)
Cordysinin D	Anti-inflammatory	Mycelium	Yang et al. (2011)
Cordysinin E	Anti-inflammatory	Mycelium	Yang et al. (2011)
Cordyceamide A	Cytotoxic	Culture supernatant	Yang et al. (2011)
Cordyceamide B	Cytotoxic	Culture supernatant	Jia et al. (2009)
Extract			
Bailing capsule	Renal protective	Mycelium	Wang et al. (2013)
Water extract	Hepatoprotective	Mycelium	Wang et al. (2012)
	Induced steroidogenesis	Mycelium	Wang et al. (1998)
	Anti-bacterial	Mycelium	Kuo et al. (2005, 2007b)
	Immunosuppressant	Mycelium	Chiang et al. (2005)
	Anti-aging	Mycelium	Ji et al. (2009)
	Improve fertilization	Mycelium	Chen et al. (1997)
	Anti-inflammatory	Mycelium	Li et al. (2009, 2012)
Ethanol extract	Anti-diabetic	Mycelium	Kan et al. (2012)
	Superoxide anion inhibition	Mycelium	Yang et al. (2011)
	Anti-inflammatory	Mycelium	Li et al. (2009)

(continued)

Table 15.2 (continued)

Bioactive compound/extract	Bioactivities	Material source	References
Methanol extract	Radiation protective	Mycelium	Lin et al. (2007)
	Immunomodulatory	Fruiting body	Kuo et al. (2001)
	Anti-inflammatory	Fruiting body	Rao et al. (2007)
	Anti-proliferative	Fruiting body	Rao et al. (2007)
	Anti-tumor	Fruiting body	Wu et al. (2007)
Ethyl acetate extract	Anti-cancer	Mycelium	Wu et al. (2007)
	Anti-tumor	Mycelium	Wu et al. (2007)
Supercritical CO_2 extract	Free radical scavenging	Mycelium	Wang et al. (2005)
	Apoptotic	Mycelium	Wang et al. (2005)
Freeze-dried powder	Anti-diabetic	Fruiting body	Lo et al. (2006)

Adopted and modified by Lo et al. (2013), Chen et al. (2013), and Chiu et al. (2016)

15.4.3 Sterols

Number of sterols have been isolated from cordyceps extracts (ergosterol, ergosterol peroxide, delta-3 ergosterol, 3-sitosterol, daucosterol, and campesterol); notably ergosterol, naturally occurring in large quantity, is a component isolated from cell membrane of this fungus that acts as a precursor to vitamin D2 and carries anti-tumor activities (Holliday and Cleaver 2008; Shashidhar et al. 2013).

15.4.4 Nucleosides

Nucleoside is considered as the primary bioactive compound of *Cordyceps* sp. which is the union of nitrogen bases and peptones. The identified nucleosides are adenine, adenosine, cordycepin, cytosine, cytidine, guanosine, guanine, hypoxanthine, inosine, thymine, thymidine, uridine, and 2′-deoxydurine. These molecules play very important role in the regulation of various physiological processes/activities in the central nervous system (Zhu et al. 1998; Li et al. 2004, 2006a, b, c, d; Russell and Paterson 2008; Winkler 2009; Shashidhar et al. 2013; Chen et al. 2013).

15.5 Artificial Culture

Cordyceps militaris, which parasitizes the pupa of Lepidoptera spp., is phylogenetically related to *Cordyceps sinensis*. The biochemical components of these two are similar; however, *C. militaris* is less expensive and more easily obtainable than *C. sinensis*. Therefore, *C. militaris* has become a model species in *Cordyceps* research (Yu et al. 2006; Cheng et al. 2012; Chen et al. 2013). The artificial culturing and stroma production of *C. militaris* have been studied in laboratory conditions on various insect pupae and larvae, most often on the mulberry silkworm (*Bombyx mori*), Eri silkworm (*Samia ricini*), and oak tasar silkworm (*Antheraea pernyi*) (Gu and Liang 1987; Liang and Gu 1987; Gu et al. 1988; Yuan 1988, 1989; Feng et al. 1990; Gong et al. 1993; Zhou et al. 2000; Li 2002; Wang et al. 2002; Pan et al. 2002; Chen and Ichida 2002; Sato and Shimazu 2002; Zhang et al. 2003; Wen et al. 2004; Liu 2004; Li et al. 2006c; Zheng et al. 2008a; Mu et al. 2010; Hong et al. 2010; Chai et al. 2010; Luerdara et al. 2015). Other insects used for artificial culturing and stroma production are *Spodoptera litura* (Sato and Shimazu 2002), *Heliothis virescens*, *H. zea* and *Spodoptera frugiperda* (Sánchez-Peña 1990), *Ostrinia nubilalis* (Liang and Gu 1987), *Mamestra brassicae* (Harada et al. 1995; Sato and Shimazu 2002), *Tenebrio molitor* (Sato and Shimazu 2002; Lin et al. 2005), *Andraca bipunctata* (Panigrahi 1995), *Philosamia cynthia* (Jiang and Xun 1996), and *Clanis bilineata* (Song 2009). Chen and Ichida (2002) observed higher infection rate and growth rate in silkworm pupae as compared larvae. Among three varieties of silkworm (Baegokjam, Daeseungjam, and Keumokjam), the Daeseungjam variety was most suitable for stroma formation of *C. militaris* (Hong et al. 2010).

Insects are very expensive and not always available in abundant quantity and also difficult to handle because they are prone to microbial contamination, so that alternative organic substrates have been tested for artificial culturing. Fortunately, cereals with the addition of some organic substances have proven to be good substitutes of insects for culturing and stroma production. Kobayasi (1941) documented stroma production of *C. militaris* on rice substrate. Many resercher used rice substrate for growing of *C. militaris* in laboratory conditions (Basith and Madelin 1968; Chen and Wu 1990; Liang 1990; Ma and Chen 1991; Sung et al. 1993, 1999; Pen 1995; Sung 1996; Wu et al. 1996; Zhang and Liu 1997; Choi et al. 1999; Zhang 2003; Li et al. 2006a, b, c, d; Lin et al. 2006; Wen et al. 2008; Chen et al. 2011a, b). Apart from this, the porosity of the fruiting medium affects mycelial growth and fruiting body yield. Porosity increases with grain size and decreases with a higher ratio of water to grain during rice medium preparation.

A ratio of rice to water (1:1 to 1:1.35) has been reported to be optimal for growth and production of stroma (Sung et al. 1999, 2002; Lin et al. 2006; Zheng et al. 2008b; Yue 2010), which also depends upon the rice cultivar and its glutinous quality. Husked rice is generally used for cultivation of *C. militaris*. Maximum fruiting body yield has been obtained with whole rice grain (Wen et al. 2008). Other organic materials used for the production of *C. militaris* stromata include bean powder, corn grain, corncobs, cotton seed coats, fragments of sunflower floral disks jowar, millet, and wheat grain (Chen and Wu 1990; Zhang and Liu 1997; Li 2002; Li et al. 2004;

Zhao et al. 2006; Gao and Wang 2008; Wei and Huang 2009). Xie et al. (2009) have observed that brown rice, malt, and soybean are also much better sources of nutrition for *C. militaris* than chemical media. Rice mixed with silkworm pupae is superior than other substrates and is now routinely used (Ren 1998; Chen et al. 2002; Shrestha et al. 2004, 2005a, b; Sung et al. 2002, 2006; Zhao et al. 2006; Jin et al. 2009).

Number of indigenous culturing methodologies for stroma production of *C. militaris*, varying from place, environment, and aim that were documented such as sawdust culture, spawn production, husked rice culture, and shaking or submerged culture (surface liquid culture, continuous culture, and repeated batch culture) (Chen et al. 2002; Ren et al. 2009; Das et al. 2010; Yue et al. 2013; Zhou et al. 2013). Sawdust culturing solid medium is inoculated with one scoop of the seed culture and maintained at 25 °C for 20 days, the fungus proliferates all over the medium (Das et al. 2010). Then, it is cultured in the dark at 20–25 °C for about 6 months; thus, knot-like fruiting bodies are formed (Das et al. 2010). Spawn culturing glass beads are added to the potato glucose medium and sterilized at 121 °C for 20 min. After cooling inoculated with several pieces of stock culture at 25 °C for a week at stationary state, the hyphae are dispersed by shaking the medium once a day. After proliferation, the fungus is inoculated into bead-free potato glucose medium and cultured for 5 days to produce a seed culture. Husked rice cultureing, the husked rice medium or wheat medium was inoculated with liquid spawn at 25 °C for 20 days, and the fungus proliferates all over the medium and was aged for about a month and harvested (Das et al. 2010). For shaking culture, the mycelia are transferred to seed culture medium by punching out about 5 mm^2 of the plate that was transferred into 250 or 500 ml shaking flask containing 50–100 ml liquid medium and incubated at 25 °C on rotary shaker (50–150 rpm) for 5–7 days, while in submerged culturing, mycelium is grown in liquid medium, which is vigorously aerated and agitated in large tank, i.e., fermenter (Das et al. 2010).

The artificial culture method comprises the following steps: preparation of the culture medium, sterilization, cooling, inoculation, culture/mycelia development, and harvesting of fruiting bodies. Numbers of solid and liquid media were utilized for culturing of *C. militaris*. Solid media such as beech wood meal, rice bran, wheat bran, husked rice, and wheat grains and liquid media, viz., potato dextrose agar (PDA), corn meal agar (CDA), malt extract agar (MEA), Ooat meal agar (OAT), and water agar (WA), and also medium containing culture bag or bottles are nowadays available in market (Sung and Shrestha 2002; Zhao et al. 2006; Masuda et al. 2007; Wei and Huang 2009; Yue 2010; Baral and Maharjan 2012). The basic culture medium comprises the following components presented in Table 15.3 (Masuda et al. 2007). Another composition of basal medium with an additive were published such as 1.25% glucose, 1.25% sucrose, 0.02% peptone, 0.0625% yeast powder, 0.025% KH_2PO_4, 0.0125% $MgSO_4$, 0.002% vitamin B_1, and 7 H_2O and natural pH at 24 °C for 192 hour (Zhao and Guo 2008), while Sheng et al. (2011) reported two times higher production of mycelia biomass by culturing on medium composed of 20% potato, 0.08% beef extract, 0.2% peptone, 0.15% KH_2PO_4, 0.15% $MgSO_4$, 2.5% glucose, 1.5% sucrose, and H_2O with natural pH at 23 °C, 130 r/min, for 4 days.

Table 15.3 Composition of basal medium (Masuda et al. 2007)

Composition of basal medium	Component concentration (g/l)
Nitrogen sources	
Peptone	2.5
Yeast extract	7.5
Carbon source	
Glucose	20
Others (diluted to 1/10 concentration of Vogel's medium)	
Sodium citrate 3 hydrate	0.28
KH2PO4	0.50
NH4NO3	0.20
$MgSO4{\cdot}7H_2O$	0.02
$CaCl2{\cdot}2H_2O$	0.01
Citric acid	0.46×10^{-3}
$ZnSO4{\cdot}7H_2O$	0.50×10^{-3}
$Fe(NH4)2(SO4)2{\cdot}6H_2O$	0.10×10^{-3}
$CuSO4{\cdot}5H_2O$	0.025×10^{-3}
H3BO3	5.0×10^{-6}
$MnSO4{\cdot}(4–5)H_2O$	5.0×10^{-6}
$Na2MoO4{\cdot}2H_2O$	5.0×10^{-6}

15.6 Industrial and Commercial Cultivation

Of the described species, only 36 have been artificially cultivated for the fruiting bodies production (Sung 1996; Li et al. 2006b; Shrestha et al. 2012). Among the artificially cultivated species, only *C. militaris* has been commercially cultivated; commercial development has focused on *C. militaris* because of its excellent pharmaceutical properties and short production period (Li et al. 2006c; Shrestha et al. 2012). *Cordyceps militaris* has been cultivated in liquid media for harvesting mycelia and on solid media for induction of fruiting bodies (Shrestha et al. 2012). Large-scale production of *C. militaris* fruiting bodies currently uses only solid media consisting of artificial substrates or insects (Shrestha et al. 2012). The most preferred method for cultivation in China is liquid fermentation, while Japan and the USA use solid substrate (cereals or grains) (Wang et al. 2009). Chinese producer uses liquid medium for fermentation which is silkworm-based liquid media that has minerals and carbohydrates added to help the mycelium grow very fast. This seems a logical choice, because this mushroom is found in nature growing on insects. Dried silkworm bodies are the by-product of an existing silkworm industry in China and have little other use. Using this liquid-based substrate, researchers/manufacturer derives a high-quality product (cordyceps). The cost of the silkworm and its constant supply make this a very cheap and efficient way to grow *Cordyceps* in China. While using silkworm as a substrate is not practicable in other countries because they do not have a silkworm industry which Chinese manufacturers have at their disposal. However, it has long been determined that using rice as a substrate to

grow *Cordyceps* would produce an inferior quality of product as rice does not help the *Cordyceps* in producing its full potential of secondary metabolites and does not produce much active ingredients in the final harvested product. And the mycelium/fruiting bodies cultivated on insects are twice as expensive to produce as biomass cultivated in artificial media (Li et al. 2007; Wang et al. 2009; Shrestha et al. 2012).

Traditional Chinese medicals are able to develop many *Cordyceps* products through modern technology. The products have been mainly focused on the following aspects: enhancing physique, anti-aging, protecting the heart, improving sleep, increasing appetite, increasing immunity, etc. For example, *C. militaris* mycelial powder and the capsule of *C. militaris* mycelial powder had been authorized as a Chinese National Drug in April 2003. Jilin Northeast Tiger Pharmaceutical Co. Ltd. reported to the State Ministry of Health to declare classes of new drugs, which have been approved and called Xinkeqi capsules (Zhou et al. 2009; Luerdara et al. 2015).

15.7 Bioprospecting Added Value to Sericulture in India

Geographically, Asia is the main producer of silk in the world and produces over 95% of the total global output. Though there are over 40 countries on the world map of silk, bulk of it is produced in China and India, followed by Japan, Brazil, and Korea. India produces four varieties of slik, the total raw silk production in 2016–2017 is 30,348 MT, of which Mulberry accounts for 21,273 MT, Tasar 3268 MT, Eri 5637 MT, and Muga 170 MT (http://csb.gov.in/publications/annual-report/). Global silkworm industry, which had been focusing on silk production, previously switched its market for supplying food supplements and raw materials for medicine (Tulasi and Viswanath 2013). Silkworm is an insect of which every part can be used for different purposes; therefore the traditional sericulture to produce only silk fabric now has been changed to functional sericulture of new paradigm to relieve the patients as well as increase the farmer income. There are a number of by-products from the sericulture industry which can be harvested and utilized in various food and medicine industries (Zhang 2002; Sarovat et al. 2003; Tulasi and Viswanath 2013). Some of the by-products from sericulture industry are:

15.7.1 Sericin

It is a water-soluble protein that is present in the silk fiber along with fibroin. Tasar sericin is unique and has many cosmeceutical, medical, and industrial applications. While processing/cooking of cocoons, this sericin gets solubilized and reaches the gutter and thus goes as waste. Ways to tap this potential resource can be focused. 1 gm crude tasar sericin can be acquired from 150–200 cocoons. The cost of 1gm pure mulberry sericin is around 10,000 rupees. Thus, the potentiality of this by-product is enormous.

15.7.2 Cocoonase

This is a proteolytic enzyme produced by the moth while emerging from the cocoon to soften it. This enzyme after molecular characterization can be mass produced using the bacterial model system. This enzyme can be helpful in softening of cocoon without interfering its natural luster. Thus, it can replace the existing chemical-based cooking procedure.

15.7.3 Chitin

This biomolecule has an enormous and diverse industrial application. It can be extracted from the dead larvae, pupae, and moths which are thrown as a waste. Suitable procedures need to be worked out for the extraction and their prosperous utilization.

15.7.4 Pupal Protein

Enormous pupal biomass goes waste every year after stifling of the cocoons for silk reeling. India imports the insect protein from China. Insect protein can be extracted and purified from pupal biomass of sericulture industry for the preparation of protein drinks, biscuits, protein powders, etc. And production of protein powder from pupal biomass or pupae itself (mixed with rice or cereals) can act as medium for *Cordyceps* mass production on large scale in India as its being used in China (Ren 1998; Chen et al. 2002; Shrestha et al. 2004, 2005b; Sung et al. 2002, 2006; Zhao et al. 2006; Jin et al. 2009). However, standardization of procedures is lacking for Indian sericulture industry. Male adults are thrown after mating and females after egg laying. These too constitute important source of medium for the *Cordyceps* mass production. For example, we are having more than 250 MT of tasar biomass (both pupae and adults) which can be utilized for the by-product development and thus can work toward the doubling of the farmer income and social upliftment of the tribal community in future.

15.8 Future Prospects and Constraints

- A systematic survey for identification of the traditional knowledge in the primary distribution region of *Cordyceps* among the local inhabitants.

- The indigenous traditional knowledge gathered by different workers related to the *Cordyceps* should be placed at a central level, from where any interested person can get all the available information.
- *Cordyceps* should be included in the list of future medicinal fungal species and considered as a crop for future research.
- Low cost and high productivity are the need of the hour. Thus the genetic manipulation from the other wild species should also be focused by the scientific community.
- The updated protocol for the highest yield in terms of profitability still needed focused work.
- The more rigorous screening for the new compounds in the identified or recently identified species should also be promoted in the research groups.
- More research and clinical evidence are highly required to support the ethnological claim information.
- The information of this important species and its benefits are highly scarce to the active silk farming.
- The government of India should include it into the business model and support financially for developing the small-scale entrepreneurship.

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Chapter 16
Synthetic Biology: A Novel Approach for Pharmaceutically Important Compounds

Rashmi, Upendra Kumar, Poonam Maan, and Priyanka

16.1 Introduction

Synthetic biology is the new emerging discipline of science which combines principles of engineering with biology to redesign a living system to produce something it would not naturally produce. The living cells will alter through recombinant DNA technology to meet specific purposes. Fungi are ubiquitous eukaryotic living organisms with diverse importance. They were interlinked with other life forms in our biosphere and have affected us in both positive and negative ways. They were diversely utilized for commercially valuable compounds since ancient times. Being the class of simple organisms yet performed biological activities to closely relate higher eukaryotic organisms made them far more valuable for commercial exploitation in the long run. A wide array of chemicals discovered and innumerable are yet to be identified (Adrio and Demain 2003).

The new emerging field of synthetic biology is arguably redirecting the drug discovery in the same way as the field of organic chemistry was once a century ago at the center of innovation in the pharmaceutical industries (Trosset and Carbonell 2015). Because of the difficulty in large-scale production of natural products through microorganisms (or other living systems), pharmaceutical industries were

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forced to redirect their interest toward simpler chemistry at the risk of increased cross-reactivity with secondary therapeutic targets and even unwanted off-targets as confirmed by recent studies in system chemical biology.

A breakthrough discovery in the 1990s made the rational-based genetic design a potential strategy for drug discovery. Microorganisms (as well as plants and others) produce secondary metabolites using gigantic biosynthetic units. These enzymatic modules can be manipulated in combinatorial fashion in synthetic cells to produce new natural product derivatives (Medema et al. 2015). The first application of synthetic biology in pharma-industry was to boost innovation in creating new chemical scaffolds that have properties similar to well-known natural products-derived human medicines, increasing the chance of being bioactive with the right pharmacological properties (Sun et al. 2015).

With the recent advanced genome editing, molecular biology, and protein engineering tools, synthetic biology has focused its aim at creating biological devices that can produce controlled products. The design of genetic circuits in synthetic biology is used in pharmaceutical research not only for bioproduction (Breitling and Takano 2015) of drugs by microorganisms but also to support the different steps of drug development. Since, fungi are themselves established cell factories in the pharmaceutical industry. The versatile chemical entities secreted by these organisms have tremendous benefits. The need of the hour is to club synthetic biology and mycology to enhance gains.

16.2 The Evolution in Synthetic Biology

Synthetic biology is a new emerging scientific discipline where engineering principles are applied to biology. Synthetic biology has gone through rapid development in the last century. The initial focus of synthetic biology was on proof-of-concept studies and now has been shifted toward the creation of complex networks in unicellular and multicellular systems and the industrial applications. Specially, the engineering of microorganisms for production of value-added small molecules such as different flavors, fragrances, and clinically relevant drugs holds great potential (Hayden 2014). In the recent years, however, the technological advancements within several fields of biology, including sequencing and other omics technologies, and gene engineering, have dramatically increased our understanding of biological systems that can be exploited within the field of metabolic engineering, where rational genetic changes are implemented in cells to improve production capabilities rather than relying on the stochastic nature of traditional mutagenesis methods. This approach holds a promising potential to change our society to a bio-based economy where any chemical in the future could be produced in a feasible way from renewable resources using microbial cell factories. A fast growth in the area of synthetic biology is well predicted in the near future with the improvement in the techniques of system design, synthesis, and optimization.

16.2.1 Synthetic Biology for Pharmaceuticals

Nowadays, drug discovery through synthetic biology is the main focus area of pharmaceutical industries. Nature is full of natural products which have been utilized as human medicines for thousands of years, but the large-scale production of natural products is difficult. Synthetic biology for drug development involves the engineering approach into biology, which alters a living cell into a biofactory for production of high-value pharmaceutically important products (Hayden 2014). The synthetic biology of medicines is further promoted by recently discovered microbial genome and metagenome sequences which have a lot of unexplored biosynthetic capacities (Wilson and Piel 2013a, b; Helfrich et al. 2014).

Among all known microbial antibiotics and similar bioactive compounds (altogether 22,500), 45% are from actinomycetes, 38% from fungi and 17% are from unicellular bacteria (Berdy 2005). Among this wealth of compounds, only about a hundred are in practical use for human therapy, with the majority being derived from actinomycetes (Berdy 2005). However, it is worth mentioning that fungi are also contributing to this group with *Penicillin* first to be utilized by pharmaceutical market now, including cholesterol-lowering statins (Barrios-Gonz and Miranda 2010), the antifungal griseofulvin (Finkelstein et al. 1996), immunosuppressant mycophenolic compounds (Stassen et al. 2007), and anticancer drugs such as taxol (Yang et al. 2014) (Fig. 16.1).

Systems biology is often divided into two different approaches: the bottom-up and the top-down approach. The bottom-up approach derives detailed models, e.g., about a biochemical pathway, and requires manual curation and a thorough prior description of the individual components of the system. The top-down approach has been integrated systems level characterization or quantifications of collections of biological entities, from high-throughput technologies, which is often referred to as omics data. Omics data can represent a multitude of different biological sources and

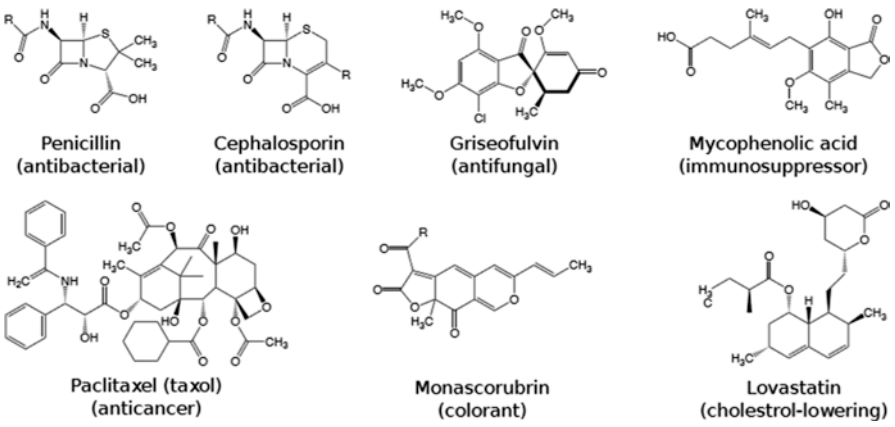

Fig. 16.1 Secondary fungal metabolites used in the pharmaceutical industry

techniques, and one of the most established omics technologies is transcriptomics, which quantifies all mRNA transcripts in a single cell or a population of cells, the transcriptome. Similarly, other types of omics technologies include genomics, proteomics and metabolomics. The inclusive and integrative nature of top-down system biology and the identification of emerging properties make it a data-driven discipline that is hypothesis generating. A common example would be the comparison of cellular responses to different conditions and applying statistical and clustering methods to extrapolate patterns in the data that define a given perturbed state of the cell described by Kitano (2002) as the systems biology research cycle.

The traditional approach to establish production processes using cell factories has been to select naturally high yielding isolates producing the compound of interest. This has historically been a successful approach in many industrial production processes of chemicals such as glutamate production by *Corynebacterium glutamicum* (Kinoshita et al. 1957) and citric acid production by *A. niger* (Currie 1917). For large-scale production of penicillin, various *Penicillium* species and isolates were screened for production. This led to the discovery of a high yielding strain, *P. rubens* NRRL 1951, which can be traced back to a moldy cantaloupe melon from the food market in Peoria, Illinois, USA. The isolate is the ancestor of all current industrial penicillin production and proved to possess a major potential for further optimization. Following decades of classical strain improvement programs through random mutagenesis, penicillin production was increased more than 10,000-fold (Thykaer and Nielsen 2003). Nowadays, an increasingly popular strategy is to transfer the pathway of interest to a platform cell factory which has been optimized for industrial production. Such organisms are advantageous since they are well characterized, and a number of gene editing and gene expression tools are established (Nielsen and Keasling 2016). In addition, existing systems biology tools such as genome-scale metabolic models (GEMs), can be exploited to easily understand the context of expression of a heterologous pathway. The yeast, *S. cerevisiae* serves as an attractive platform for secondary metabolite production and has been successfully used to express heterologous pathways of fungal PKs (Naesby et al. 2009; Rugbjerg et al. 2013) and NRPs (Awan et al. 2017).

The production of anti-malarial, semi-synthetic drug artemisinin is the first application of synthetic biology in the pharmaceutical industry and mostly used as a model drug for practice of synthetic design and assemble biological modules, devices, and systems. Originally, this drug was obtained from a plant, *Artemisia annua*, but for large-scale production can be produced in different heterologous hosts by conjoining metabolic engineering and synthetic biology (Paddon et al. 2013). The microorganisms were metabolically engineered to produce artemisinic acid (chemical precursor of artemisinin), followed by synthetic organic chemistry to produce semi-synthetic artemisinin. The semi-synthetic artemisinin is indistinguishable to natural artemisinin and known as semi-synthetic because its precursors are produced biologically and only a few final steps are achieved by organic chemistry.

To understand the semi-synthetic production of artemisinin, a thorough study of its production in *A. annua* is required (Bertea 2005; Brown 2010) (Fig. 16.2).

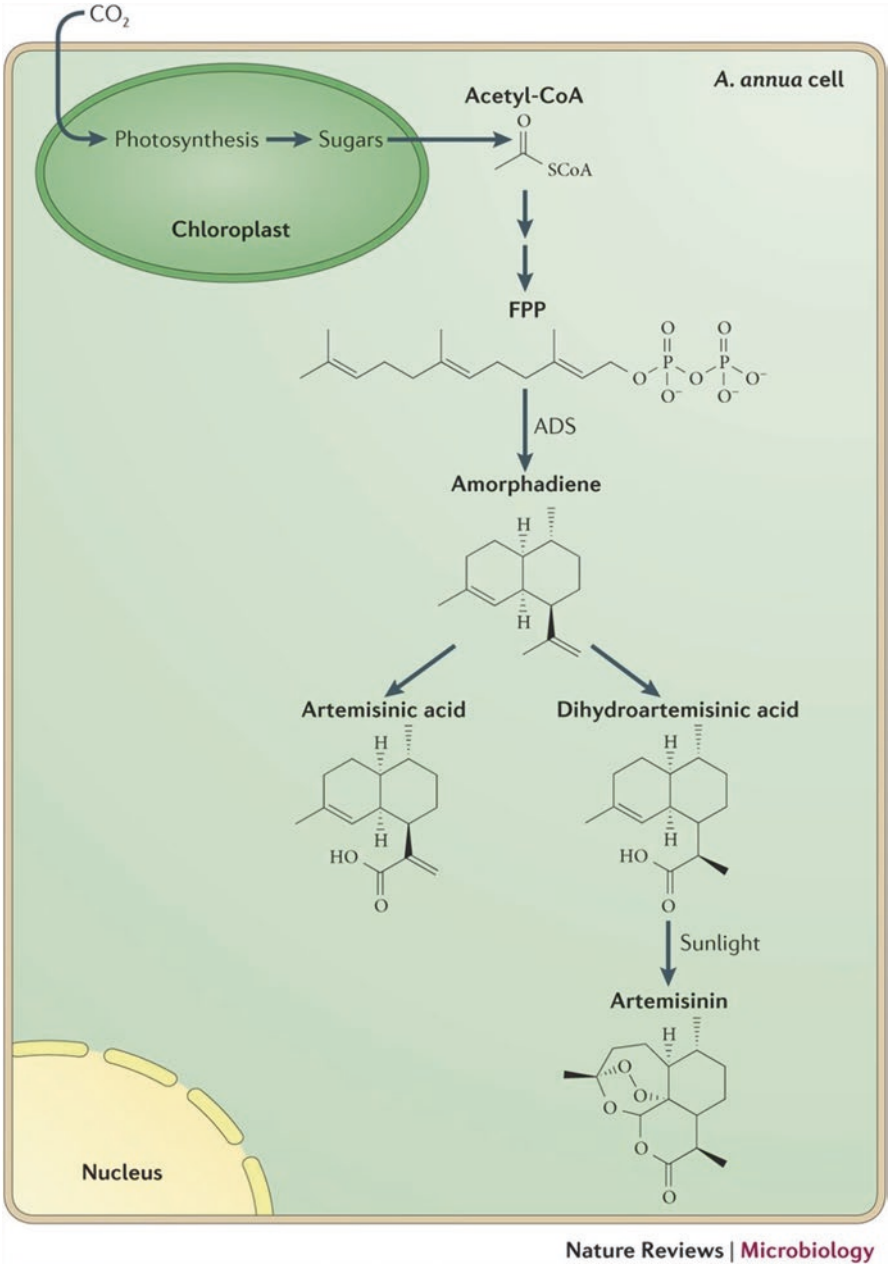

Fig. 16.2 Artemisinin biosynthesis pathway in the plant *Artemisia annua*. (Source: Paddon and Keasling 2014)

Artemisinin is an isoprenoid molecule containing 15 carbon atoms and a lactone endoperoxide (White 2008). *A. annua* produces acetyl-CoA (2C) from sugars; then it enters in the mevalonate pathway and gives rise to the farnesyldiphosphate (FPP) (15C). Farnesyldiphosphate is converted to amorphadiene (15C isoprenoid hydrocarbon) by the enzyme amorphadiene synthase (ADS) (Bouwmeester et al. 1999; Covello et al. 2007). Amorphadiene is oxidized into artemisinic acid and dihydroartemisinic acid (Paddon et al. 2013). Artemisinin is derived from dihydroartemisinic acid spontaneously in the sunlight (Bertea et al. 2006; Brown 2010).

16.2.2 Synthetic Biology in Drug Development

16.2.2.1 Artemisinin Production

The main objective of semi-synthetic artemisinin production process was to engineer *E. coli* or other microorganisms to produce high titer and yield of artemisinin precursors such as artemisinic acid, followed by chemical conversion to artemisinin. Artemisinic acid was produced by *E. coli* through fermentation and then synthetic organic chemistry was used to produce artemisinin from artemisinic acid. However, in engineered *E. coli*, it was difficult to oxidize amorphadiene to artemisinic acid; this encouraged the use of *S. cerevisiae* in place of *E. coli* for production of artemisinic acid. The engineered *S. cerevisiae* strain was very efficient in production of amorphadiene as it can produce 40 g per L amorphadiene in comparison to *E. coli* which can produce 25 g per L. The process of artemisinin production involves following developmental stages:

16.2.2.1.1 Stage 1: Amorphadiene Synthesis in *E. coli*

Synthesis of isoprenoids follows two different pathways, the mevalonate and 1-deoxy-d-xylulose 5-phosphate (DXP) pathway. The mevalonate pathway takes place in eukaryotes as well as in some prokaryotes while DXP pathway takes place in bacteria and plant chloroplasts (Zhao et al. 2013). The genes that encode isoprenoid synthases and key enzymes of the DXP pathway were overexpressed for enhancing the flux through the pathway, this was resulted into enhancement in the production of heterologous isoprenoids (C10 and C20). However, the low titer of terpenes is produced through this strategy which may be because of synthesis inhibition in native host (Reiling et al. 2004). However, production was increased by expressing the yeast mevalonate pathway into *E. coli*. A synthetic version of the natural amorphadiene synthase (ADS) (Martin et al. 2003) was also expressed. Amorphadiene synthase is required for conversion of endogenously produced FPP to amorphadiene.

The mevalonate pathway in *E. coli* was expressed by two plasmids encoding mevT operon and mevB operon. These two operons (mevT operon and mevB

operon) belong to two different processes; top pathway and bottom pathway, respectively. mevT operon consists of three genes, i.e., atoB, ERG13, and tHMG1, required for the conversion of acetyl-CoA to mevalonate. mevB comprises five genes, i.e., idi, ispA, MVD1, ERG8, and ERG12, that are responsible for conversion of mevalonate to FPP. mevT operon is overexpressed, and its overexpression is associated with the low growth of microorganism (Pitera et al. 2007); this was due to the imbalanced expression of enzymes atoB, ERG13, and tHMG1 that were encoded by the mevT operon. This leads to the accumulation of an unknown intermediate that inhibited the growth. Then, *S. cerevisiae* was used as bio-factory to test its efficiency for production of artemisinin precursors.

16.2.2.1.2 Stage 2: *Saccharomyces cerevisiae* as Production System

Amorphadiene is oxidized by a cytochrome P450 enzyme located in the trichomes of *A. annua* (Bertea 2005). *S. cerevisiae* was used as a host to express this enzyme (P450 (CYP71AV1) along with its cognate reductase (CPR1) for assessing the functionality of this enzyme. This transformed stain of *S. cerevisiae* was capable of producing 150 mg per L amorphadiene which was much lower than that in *E. coli* (25 g per L). It is not an easy task to express eukaryotic P450 in *E. coli*, and production of high titers of artemisinic acid was also in doubt. After considering a number of possibilities, the researchers concluded that *S. cerevisiae* was the superior organism.

Although some oxidized intermediates of amorphadiene (such as artemisinic alcohol) could be produced by *E. coli*, *Escherichia coli* could produce only more than 1 g per L artemisinic acid by expression of CYP71AV1, at 20 C which is an unsuitable temperature for industrial fermentation. While in *S. cerevisiae* 2.5 g per L artemisinic acid was produced by optimizing the production pathway and further improvement in titer was possible using an alternative yeast strain.

Use of an Alternative Yeast Strain The *S. cerevisiae* strain S288C was used initially for the production of artemisinic acid. The major drawback with this strain was that it sporulates poorly (Ben-Ari et al. 2006) and strain construction is not easy. Industrial fermentation with this strain is also difficult because of little information available about it. By contrast, another strain *S. cerevisiae* CEN.PK2 was proved to be desirable because it sporulates profoundly and has characteristics required for industrial fermentation (vanDijken et al. 2000). Amorphadiene was produced in fivefold higher concentrations by using the engineered strain *S. cerevisiae* CEN. PK2 as compared to the original S288C-derived strain. StrainCEN.PK2 was capable of producing 40 g per L amorphadiene by further optimization of fermentation process (Westfall et al. 2012). Although the amorphadiene production was increased, it did not result in the enhancement in artemisinic acid which was tenfold lower in production level.

Improvement in Artemisinic Acid Production Level It was observed that the engineered CEN.PK2 strain expressing CYP71AV1 for artemisinic acid production goes through a severe decrease in viability. This decrease in viability did not occur with the strains that lacked CYP71AV1 and produce amorphadiene (Paddon et al. 2013). Artemisinic acid production in S288C-derived strain is associated with the induction of transporter gene expression in high level (Ro et al. 2008). Transcript analysis indicated that cells expressing CYP71AV1 undergo severe oxidative stress. CYP71AV1 and its reductase (CPR1) were expressed in high concentrations in the yeast strain. The expression of CPR1 was reduced later on, which led to improved viability but associated with reduced production of artemisinic acid.

The cytochrome P450 enzymes interact with cytochrome b5. Cytochrome b5 from *A. annua* was transferred and expressed in the CEN.PK2 production strain due to this, the production level of artemisinic acid was enhanced but it was still lower than the target concentration of 25 g per L (Paddon et al. 2013). This strain produced a highly reactive oxidation intermediate, artemisinic aldehyde which is considered as toxic and creating difficulties in producing the amount of artemisinic acid in yeast. The production of artemisinic acid was increased considerably by the expression of *A. annua* artemisinic aldehyde dehydrogenase (ALDH1) (Teoh et al. 2009). Another gene that encodes for an NAD-dependent artemisinic alcohol dehydrogenase (ADH1) was also isolated from *A. annua* and expressed together; this resulted into the highest concentration of artemisinic acid that had been achieved so far.

16.2.2.1.3 Stage 3: Chemical Conversion of Artemisinic Acid to Artemisinin

A four-step chemical process was developed for conversion of purified artemisinic acid to artemisinin (Paddon et al. 2013). In the first step, artemisinic acid was reduced to dihydroartemisinic acid. Then, esterification of the -COOH moiety on dihydroartemisinic acid was done followed by the generation of singlet oxygen. Singlet oxygen was responsible for the production of 3-hydro-peroxide and acid-catalyzed Hock fragmentation and rearrangement. Ultimately, artemisinin is produced in the presence of molecular oxygen. This process can be scaled up by using batch reactors at manufacturing sites that are not much expensive to operate.

16.2.2.2 Taxol Production Using Endophytic Fungi

Taxol® (generic name Paclitaxel), a plant-derived anticancer drug, widely used against breast, ovarian, and lung cancer, was originally isolated from the Pacific yew, *Taxus brevifolia*. The limited supply of the drug prompted research to find alternative sources such as chemical synthesis tissue and cell culture of the *Taxus* species both of which are expensive and yield low levels. Thus, a sustainable, economical and unconventional alternative source of Taxol® such as endophytic fungi has been actively researched. This discovery was projected as the dawn of a new era

of endophyte biotechnology with billions of dollars' worth of global market for Taxol® already in place, and agreements were immediately underway among leading pharmaceutical companies to explore the possibility of fungal Taxol® production through industrial fermentation (Flores-Bustamante et al. 2010). The inability of the fungus to show reproducible high titer yields of Taxol® in axenic cultures, thus not amenable to industrial scale-up, led to the disappointing failure in delivering the promises of this highly heralded discovery. However, Taxol®-producing endophytic fungi harbored in other Taxus species and even in non-Taxus plants including many angiosperms, have been regularly reported (Flores-Bustamante et al. 2010; Hao 2013). At the present time, around 200 endophytic fungi belonging to more than 40 fungal genera from several different orders representing mostly *Ascomycota* and *Deuteromycota*, with only a few from *Basidiomycota* and *Zygomycota*, have been reported to produce Taxol® (Flores-Bustamante et al. 2010; Hao 2013). Many endophytic fungi are added to the list every year underlining the fact that only a tiny fraction of an estimated one million (or more) endophytic fungal species has been cultured and screened (Suryanarayanan et al. 2009). Undeniably, none of these discoveries have been successfully translated into industrial bioprocesses so far.

The molecular pathway of Taxol® biosynthesis in different *Taxus* plants has been well characterized at both native and recombinant levels with the discovery of close to 20 different enzymatic steps (Fig. 16.3) spatially organized in plastids, endoplasmic reticulum, and cytosol (Jennewein and Croteau 2001; Walker and Croteau 2001; and Croteau et al. 2006). However, the molecular signature of Taxol® biosynthesis in Taxol® producing endophytic fungi remains largely ill-defined. Several groups have independently attempted to screen many of these fungi through PCR-based approaches to seek these biosynthetic blueprints using primers designed from the Taxol® biosynthesis gene sequences of different *Taxus* plants available in the databases (homology-based approach) (Flores-Bustamante et al. 2010; Hao 2013; Staniek et al. 2009; Garyali et al. 2013; Xiong et al. 2013). Indeed, reports on the PCR amplification and cloning of many genes of this pathway from several Taxol® producing endophytic fungi (Table 16.1) facilitate a decisive re-evaluation of their 'true' biosynthetic potential, and in turn their potential as alternative and sustainable sources of Taxol®.

Genetic engineering of endophytic fungi known to produce Taxol®, both by gene overexpression and random mutagenesis coupled with genome shuffling, have been attempted in only a very limited number of fungal isolates. In *Ozonium* sp. EFY-21 isolated from *T. chinensis* var. *maire*i, overexpression of Taxus TS gene under a fungal-specific promoter resulted in about fivefold increase in Taxol® production as compared to control (Wei et al. 2012). Multiple mutagenesis of *Nodulisporum sylviforme* provided the strain NCEU-1 from which three hereditarily stable strains were obtained by mutagenesis. Protoplasts (round fungal cells generated from spores and lacking the cell wall) generated from these and fused randomly finally led to three strains that showed an increase in Taxol® yield by 31, 64, and 45% over the control, respectively (Kai et al. 2008). The reported Taxol® pathway metabolic engineering approaches in *Escherichia coli* (Ajikuar et al. 2010;

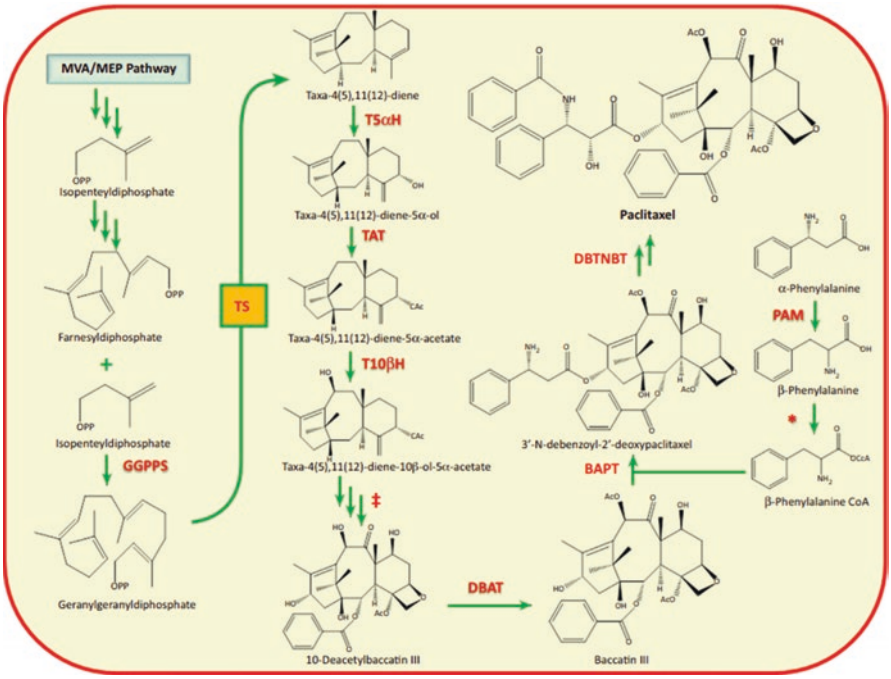

Fig. 16.3 Prevalent consensus biosynthetic route for Taxol® in Taxus species. Abbreviations: *MVA* mevalonic acid, *MEP*, 2-C-methyl-D-erythritol-4-phosphate, *GGPPS* geranylgeranyldiphosphate synthase, *TS* taxa-4(5),11(12)-diene synthase that catalyzes the committed step of this pathway, *T5aH* taxa-4(5),11(12)-diene-5a-hydroxylase, *TAT* taxa-4(5),11(12)-diene-5a-ol-O-acetyltranseferase, *T10bH* taxane-10b-hydroxylase, *z* 'oxytane ring' formation and branch migration enzymes including taxane 2a-Obenzoyltransferase (T2BT or DBBT = debenzoyltaxane-20-a-O-benzoyltransferase) as well as C-13 hydroxylation and steps taking pathway flux towards non-Taxol1-type molecules, *DBAT* 10-deacetylbaccatin III-O-acetyltransferase, *BAPT* baccatin III 13-O-(3-amino-3-phenylpropanoyl) transferase, *DBTNBT* 30-N-debenzoyl-20-deoxytaxol-Nbenzoyltransferase which follows hydroxylation in the side chain by an unknown enzyme, *PAM* phenylalanineaminomutase, *, b-phenylalanine coenzyme A ligase. Multiple arrows imply more than one biosynthetic step. The Taxol® biosynthetic pathway is proposed to have about 20 different enzymatic steps in Taxus plants

Huang et al. 2001; Meng et al. 2011) and *Saccharomyces cerevisiae* (Engels et al. 2008; DeJong et al. 2005) have mostly focused on taxadiene engineering. Reported attempts to engineer the Taxol® biosynthetic pathway beyond taxadiene encountered metabolic bottlenecks as observed by the total absence or insignificant yields of any intermediate beyond taxadiene. This was shown for seven consecutive gene transfers in *S. cerevisiae*, which is the highest number of steps (for the Taxol® pathway), engineered in a heterologous host so far (Dejong et al. 2005).

Notwithstanding these unsuccessful endeavors, however, the several hundred milligrams per liter (reaching 1 g/l) yields of taxadiene obtained in few such attempts together with reports of biotransformation of intermediate taxanes by several microbial enzymes provide some strategies worth exploring to realize sustained Taxol®

Table 16.1 Taxol® biosynthetic pathway genes reported from endophytic fungi under the SMF

Gene name	Molecule type	Fungus	Host plant	GenBank acc. no.	Fermentation method	References
TS	cDNA	*Fusarium solani*	*Taxus celebica*	HM113487	SMF	–
TS	gDNA	*Taxomyces andreanae*	*Taxus brevifolia*	–	SMF	Staniek et al. (2009)
TS	gDNA	*Fusarium redolens*	*Taxus baccata subsp. wallichiana*	–	SMF	Garyali et al. (2013)
TS	gDNA	*Gibberella intermedia*	*Taxus x media*	KC337345	SMF	Xiong et al. (2013)
TS	gDNA	*Mucorrouxiaus*	*Taxus chinensis*	–	SMF	Miao et al. (2009)
TAT	cDNA	*Ozonium* sp. BT2	*Taxus chinensis* var. *mairei*	AY960682	SMF	–
10βH	gDNA	*Ozonium* sp. BT2	*Taxus chinensis* var. *mairei*	AY836677	SMF	Guo et al. (2006)
	cDNA			AY907826		
13αH	cDNA	*Fusarium solani*	*Taxus celebica*	EF626531	SMF	Chakravarthi et al. (2008)
DBAT	gDNA	*Fusarium solani*	*Taxus celebica*	GU392264	SMF	–
DBAT	gDNA	*Cladosporium cladosporioides* MD2	*Taxus x media*	EU375527	SMF	Zhang et al. (2009)
DBAT	gDNA	*Aspergillus candidus* MD3	*Taxus x media*	EU883596	SMF	Zhang et al. (2009)
DBAT	gDNA	*Fusarium redolens*	*Taxus baccata* subsp. *wallichiana*	–	SMF	Garyali et al. (2013)
BPAT	gDNA	*Taxomyces andreanae*	*Taxus brevifolia*	–	SMF	Staniek et al. (2009)
BPAT	gDNA	*Colletotrichum gloeosporioides*	*Taxus x media*	KC337344	SMF	Xiong et al. (2013)
BPAT	gDNA	*Guignardia mangiferae*	*Taxus x media*	KC337343	SMF	Xiong et al. (2013)
BPAT	gDNA	*Fusarium redolens*	*Taxus baccata* subsp. *wallichiana*	KC924919	SMF	Garyali et al. (2013)

Abbreviations: *TS* taxa-415,11(12)-diene synthase, *TAT* taxa-4(5),11(12)-diene-5α-ol-O-acetyl transferase, *T10βH* taxane-10β-hydroxylase, *T13αH*, taxa-4(5), 11(12)-diene-13α-hydroxylase, *DBAT* 10-deacetylbaccatin III-O-acetyl transferase, *BAPT* baccatin III 13-O- (3-amino-3-phenylpropanoyl) transferase, *SMF* submerged fermentation, *cDNA* complementary DNA, *gDNA* genomic DNA

supply using endophyte (and related microbial) biotechnology. This would be especially interesting when supplemented with contributions from heterologous hosts and other optimization methodologies and tools such as intracellular compartment optimization, storage and efflux modulation, and control of pathway regulatory elements. However, delineation of the molecular mechanisms of Taxol® biosynthesis and regulation thereof remains a prerequisite for all such endeavors. Most notably, as seen from the Taxol® biosynthetic pathway of *Taxus* sp., there seems to be an obvious hurdle in engineering such a lengthy and complex pathway in its entirety in heterologous hosts. Transformation of genes of the entire pathway is a challenge and more importantly, regulation of Taxol® production encompassing epigenetic modulation and signaling crosstalk itself remains a poorly understood topic. Taxol®-producing endophytic fungi, therefore, still present a viable and long-term target, despite many unanswered questions.

16.2.2.3 Production of Other Pharmaceuticals

Artemisinin is the first pharmaceutical agent produced at industrial scale by using both metabolic engineering and synthetic biology. Semisynthetic artemisinin has been approved by the WHO as it is functionally similar to the natural drug obtained from *A. annua* 55. It is predicted that similar techniques can be used to produce many other pharmaceutical products. Production of anticancer drug Taxol (Jiang et al. 2012), farnesene (Zhu et al. 2014) and HIV drug prostratin has already been attempted through synthetic biology. Moreover, several precursors of useful alkaloids which are used as antioxidants, analgesics, and muscle relaxants have been produced in biofactory of *E. coli* and *S. cerevisiae* (Hawkins and Smolke 2008; Minami et al. 2008). Polyketides are a large class of important natural products, e.g., erythromycin, epothilone, and FK-506 are also produced using this technology.

A significant achievement in the recombinant production of polyketides in *E. coli* (that naturally does not produce any) was the engineering of the pathway that encodes the production of 6-deoxyerythronolide B (6dEB) (Pfeifer et al. 2001). This strain was created by optimizing precursor production, enzyme engineering and destroying catabolic pathways. Subsequent optimization of the strain has improved the level of 6dEB production to levels obtained from optimized *S. coelicolor* strains (Pfeifer et al. 2002). Building on this work, heterologous production of several other important polyketides or their precursors has been achieved recently, namely, the anti-cancer drugs epothilone C and D (Mutka et al. 2006) and ansamycin precursors (Rude and Khosla 2006; Watanabe et al. 2003), aklanoic acid (precursor to several antitumor polyketides, e.g., doxorubicin and aclacinomycin A) (Lee et al. 2005), and aromatic bacterial polyketides (Zhang et al. 2008).

16.3 Drivers and Constraints for Adoption/Future Challenges

Pharmaceuticals have inspired some of the earliest success stories of synthetic biology for two main reasons: Firstly, small molecule drugs in current use (from aspirin to artemisinin) are very often derived from natural products, so that a return to microbial production systems is seen as relatively straightforward, and secondly, many natural biosynthetic pathways show a surprising level of built-in modularity at many levels, which can be exploited by the engineering approaches of synthetic biology(Frasch et al. 2013; Medema et al. 2011). The synthetic biology of pharmaceuticals is further inspired by the recent avalanche of microbial genome and metagenome sequences which revealed an unexpected richness of unexplored biosynthetic capacities in almost every genome analyzed (Wilson et al. 2014; Helfrich et al. 2014). The main driver for the adoption of this technique is in its great technical potential. Besides the broad applicability, they show specific technical advantages when compared to 'older' techniques.

The second main driver is the economic benefit. The use of a new technique makes the process faster which lowers production costs. In principle the commercial development of synthetic biology could be driven by advantages at the technological level or the economic level. At another level, synthetic biology also contributes to the fundamental understanding process (Andrianantoandro et al. 2006) that can be further tapped by scientific endeavors. However, it is also possible to anticipate technical constraints (current efficiency) as majority of work performed in synthetic biology is in basic science rather than applied science with exceptions mentioned. Majority of projects were centered on developing new experimental and computational tools, using synthetic biology to understand how organism work or to generate minimal cells that can be counted as time constraint as it is also evident in case of semi-artiseminin where it took 10 long years.

Other constraints include the range of hosts to be used for generating pharmaceuticals which can be easily overcome by rapid development in the field, e.g., in *Streptomyces orinici*, silent biosynthetic pathway was awakened by refracting strategy of synthetic biology by Shao et al. (2013) by removing all negative control of the spectabilin cluster and replacing it with system of constitutive and inducible heterologous promoters in a plug-and-play scaffold. This can help in straightforward production of end products at detectable levels amenable for analysis and further optimization. Industrial *Streptomyces avermitilis* (Komatsu et al. 2013) and *Pseudomonas* strains (Nikel 2014) are the new hosts for biotechnological applications based on the increasing ability of synthetic biology to engineer such as non-classical mode organisms. The most attractive production host, i.e., *E. coli*, has also demonstrated the ability of heterologous expression of huge molecular assembly lines required for pharmaceutically interesting products by Jaitzig et al. (2014). Further, CRISPR-*Cas* system and related technologies allowed achieving genome engineering in multicellular organisms, even in plants (and plant cell cultures) for

production of pharmaceutical compounds (Staniek et al. 2013; Wilson et al. 2014). Other constraints like posttranslational modification on the expression of proteins are largely unexplored.

16.4 Conclusion and Future Prospects

Pharmaceuticals industry basking new glory under synthetic biology as discussed above. New molecular compounds as well as hosts were formed and manipulated for human benefit. Engineered systems are rapidly becoming a reality which is based on advances in our ability to edit genome and identify and optimize biosynthetic building blocks. This can help in creating a library of new pathways and novel compounds. But at the same time, ethical issues centered about the complete engineering of a new living organism or redesigning of existing species caught negative attention. It may or may not create problem but the danger of evolving new virulent strains always crossed in mind. Anyways, bolder initiatives are needed in funding for using this technology as it will be benefitted by the advancement of computational and engineering technology which in the future will move many more examples.

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